Methane Production in Peatlands

by

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Abstract

Methane emissions from peatlands have been researched for decades, although our understanding of methane production at microbial scales is still limited, and this may hamper our ability to predict methane emissions from site-to regional- to global scales. Here, a multi scale approach is used to expand on current knowledge related to the controls and the microbial community responsible for methane production within peatlands. For the first time, an analysis of methane production from a global, coordinated sampling effort was done using a standardized laboratory methodology. Site pH and plant communities were shown to be the best predictors of methane production at the global scale, while peat organic chemical characteristics and abiotic factors including temperature, moisture, and nutrient concentrations, were also shown to be important. Around 5% of samples showed disproportionately high methane emissions compared to CO_2. The second research project narrowed focus to a regional scale: peatlands in the Sudbury, ON region were evaluated to assess the role historic and contemporary smelting activities, and subsequent metals and sulfur deposition, have had on the methanogen community composition and methane production. In comparison to most peatland studies, the methanogens present in impacted sites were largely unclassified at the order level and production of methane was dramatically decreased compared to the reference locations. The third research project in the thesis focused even more exclusively at the microbial scale: enrichments of peat were used in an attempt to isolate novel methanogens. While no pure culture isolates were obtained, novel methanogens at the genera and species level where obtained from five of the seven known methanogenic archaeal orders. The case is made that obtaining enrichment (mixed) cultures is an important, underused methodology for discovering and learning about novel methanogens, which have very tight, perhaps inseparable, syntrophic relationships with other anaerobic microbes.

Combining culture techniques with modern sequencing technologies was explored as a way forward in obtaining novel species genomes and related growth conditions. Taken together, the overall controls on methane production at global and local scales are commonly peat pH, plant community composition, and peat quality, while the methanogen communities responsible for methane production remain largely unknown and underrepresented in culture collections highlighting the need for further enrichment and isolation work.

Keywords

Methanogens, methane, peatlands, *mcrA*, greenhouse gases, enrichments, symbionts, anaerobic, culturing, next gen sequencing, mining impacts

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Co-Authorship Statement

This thesis was written with me as the primary author, however co-authors were involved in varying aspects of this work. Individual contributions to research chapters are outlined below, and any oversights or errors throughout the thesis are my own.

Chapter 2 Patterns and predictors of methane production across peatlands globally

This chapter was a part of the Global Peatland Microbiome Project and involved substantial work from numerous contributors. The conceptual design was led by Erik Lilleskov (US Forest Service, PI), Nathan Basiliko, and 3 other co-Is, and PDF, Louis J. Lamit (Michigan Technological University, USFS) contributed greatly to the procurement and organization of samples. I contributed to the local sampling effort, data collection and sample preparation, and was wholly responsible for the methane flux measurements and manuscript draft construction.

Chapter 3 Altered Methanogen Communities and Methane Production in Northern Peatlands Following Long-term Smelter Deposition of Ni, Cu, and S

I completed the primary research, experimental design, data collection and analysis, and draft form of this manuscript. Graeme Spires (Laurentian University) and Shaun Watmough (Trent University) both contributed to the analysis of samples in their labs for ICP-MS elemental data and CNS data, respectively. While all co-authors, including Suzanna Bräuer (Appalachain State University), Nathan Basiliko, and Peter Beckett (Laurentian University) provided useful edits and comments leading to the final version of this chapter.

Chapter 4 Environmental enrichments for novel methanogens: Approaches for obtaining uncultured organisms in the age of rapid sequencing

I conducted the field and lab work for this chapter as well as wrote the original draft. The conceptual design and methodological process was influenced greatly by the skills and knowledge of Suzanna Bräuer (Appalachain State University). Nathan Basiliko (Laurentian University) and Suzanna Bräuer added useful edits and comments during manuscript preparation.

Acronyms

ΔG°	Gibbs free energy
ANOVA	Analysis of variance
AS	Ashigami (-M middle; -D deep)
BL	Broder Lake (-M middle; -D deep)
bp	Base pair
C	Carbon
CCA	Canonical correspondence analysis
CF	Cartier Forest (-M middle; -D deep)
CH_4-C	Carbon as methane
CL	Cartier Lawn (-M middle; -D deep)
Co	Cobalt
CO_2	carbon dioxide
CO_2-C	Carbon as carbon dioxide
Cu	Copper
CW	Clear Water (-M middle; -D deep)
DL	Daisy Lake (-M middle; -D deep)
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
F430	Cofactor of *mcrA*
Fe	Iron
FTIR	Fourier transformed infrared spectroscopy
GC	Gas chromatograph
GHG	Greenhouse gas
GPS	Global positioning system
GWP	Greenhouse warming potential
ICP-MS	Inductively coupled plasma mass spectrometry
IJSEM	International Journal of Systematic and Evolutionary Microbiology
LL	Long Lake (-M middle; -D deep)
LOI	Loss on ignition

LU	Laurentian (-M middle; -D deep)
mcrA	Encoding for the *A* subunit of the methyl coenzyme-M reductase
MG	Matagamasi (-M middle; -D deep)
Mo	Molybdenum
n	sample number
N	Nitrogen
NA	Missing/non-measured values
NCBI	National Center for Biotechnology Information
NEP	Net Ecosystem Productivity
Ni	Nickel
NPP	Net Primary Productivity
NTC	No template control
OM	Organic matter
OTU	Operational taxonomic unit
P	phosphorus
PCA	Principal component analysis
PCR	Polymerase chain reaction
Q_{10}	Reaction rate change with 10 °C increase
qPCR	Quantitative PCR
R	Programming language used for statistics and plotting
RC	Rock Cut (-M middle; -D deep)
RCII	Rice cluster II
rRNA	Ribosomal ribonucleic acid
S	Sulfur
SSU rRNA	Small subunit ribosomal ribonucleic acid
TMA	Trimethylamine
TRFLP	Terminal restriction fragment length polymorphism
WT	water table

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Chapter 1 Introduction

1.1 Background

1.1.1 Peatlands

In many landscapes, wetlands are the transitional ecotypes between terrestrial and aquatic ecosystems. In general three main characteristics define wetlands including seasonal or permanent saturation, distinct soil composition compared to surrounding upland or aquatic sediments, and vegetation adapted to, or tolerant of saturated soils (Mitsch and Gosselink, 1986). Globally, wetlands account for ca. 6.2-7.6% of land area (Lehner and Döll, 2004), but contribute a disproportionate amount of the greenhouse gas methane emissions; an estimated 55-89% of naturally emitted global atmospheric methane comes from wetlands (Schlesinger and Bernhardt, 2013). In Canada wetlands cover an estimated 127.2 million ha or 14% of the land area (Tarnocai, 1984), and serve many critical ecosystem functions. Wetlands can act as "filters" between nutrient rich terrestrial systems and nutrient poor aquatic systems, are key players in global nutrient cycling (Reddy et al., 1999; Jordan et al., 2010), and represent large stores of terrestrial carbon (C) containing an estimated 50% of global soil C (Tarnocai et al., 2009).

Peatlands (synonymous with *mires* in Europe) are a subset of the broader classification of wetlands where vegetation growth (net primary productivity; NPP) is relatively slow, subsequently buried, and partially decomposed in an active oxic/anoxic boundary layer termed the *acrotelm*. As biomass accumulates it becomes compacted under permanently saturated conditions in the *catotelm* (Brinson et al., 1981), leading to accumulation of organic matter or

"peat". Saturated soils that are characteristic of peatlands (as well as other wetland types) contribute to a number of distinct characteristics including low to absent soil oxygen (O_2). This is primarily driven by the difference in diffusion rates in water vs. air, and greatly impedes aerobic decomposition, thus leading to organic C buildup. Thus, although peatlands are important global sources of methane, they are simultaneously long-term net sinks for carbon dioxide (CO_2). While partially decomposed organic matter in peatlands is effectively "protected" under anoxic conditions due to saturation and much slower anaerobic decomposition processes, it is a prime target for future and rapid decomposition under altered nutrient, pH, oxic conditions brought about by climate change induced drought.

On a global scale peatlands cover ca. 0.5 -3 % of the land area (Gorham, 1991; Lehner and Döll, 2004), however Canada has a disproportionately high amount of peatlands covering ca. 12% of the land area (National Wetlands Working Group, 1988). Peatlands are the dominant wetland class in Canada with fens and bogs accounting for 91% of all wetlands, amounting to 23 trillion m^3 of peat weighing 507 billion tonnes (Tarnocai, 1984). Ontario is one of the most wetland-dense provinces accounting for 20% of Canada's total peatland area and 25% of the land area in the province. The majority of Canadian peatlands began to develop following the Wisconsin glaciation and basal dates put the earliest peatland development at around 5,600-10,100 years ago in the arctic (Zoltai and Tarnocai, 1975) and closer to 3,000 years ago in certain boreal regions (Tarnocai, 1978; Dionne, 1979), and older in more southern sites (Roulet et al., 2007). While there are some unifying characteristics of peatlands, a large amount of abiotic and biotic variation exists across Canadian peatlands and peatlands globally.

Bogs

Bogs (*muskeg* in parts of Alaska and Canada) are peatlands that have little to no hydrologic inflow to the system from ground or surface waters and are often dominated by *Sphagnum* mosses with ericaceous shrubs, black spruce (*Picea mariana*) and/or tamarack (*Larix laricina*) commonly present. In northern latitudes these are often referred to as *ombrotrophic* literally meaning, "rain fed" due to the lack of inflow into these vast systems, and dependence of plants on atmospheric deposition of nutrients (Mitsch and Gosselink, 1986). While nutrients are limiting in most wetlands, bogs in particular have very tight nutrient cycles. For example, phosphorus (P) has been shown to have retention times of 220 years and a residence time of 10 hours for labile forms (Chapin et al., 1978). Under current classification there are 18 forms of bogs in Canada based on variation in surface form, water type and/or morphology of base mineral soil (National Wetlands Working Group, 1988).

Fens

Fens are the second type of peatland and often have sedge, grass, and/or shrubs interspersed with mosses. They are a distinct class of wetlands from bogs in that they commonly have hydrologic connectivity to ground and/or surface water (Mitsch and Gosselink, 1986). Groundwater flow means that in general nutrients are less limiting in fens compared with bogs, and are termed minerotrophic. Fens are classified into 17 forms in Canada based on variation in surface form, water type and/or morphology of base mineral soil, just as with bogs (National Wetlands Working Group, 1988). A gradient (pH and Ca/Mg) within the fen class and forms also exists leading to the common type designation of poor, intermediate, or rich.

Tropical

In addition to northern latitude peatlands, tropical regions (especially in SE Asia and Oceania) are common. Peat in these regions is markedly different in that they are formed from primarily forest litter, which is subsequently submerged in anoxic condition and resists decomposition. In these hot environments, water table position is critical in maintaining anaerobic conditions and subsequently peat formation. Of particular concern for tropical peatland is the heavy amount of land use change and deforestation offering in these regions, typically a result of conversion to agricultural systems which causes dramatic decreases in the soil C stocks (Osaki and Tsuji, 2016).

1.1.2 Global Carbon Budget

On a global scale, atmospheric methane concentrations are much lower than CO_2 (1.83 ppm compared to >400 ppm, respectively) but have been increasing since the 1800's at an exponential rate faster than that of CO_2 (Schlesinger and Bernhardt, 2013). While concentrations are lower, effects of methane on global warming potential are, on a molecule-to-molecule basis, more significant than that of CO_2, with 100-year radiative forcing of 25-35x that of CO_2 (Lashof and Ahuja, 1990; Shindell et al., 2009). This means that small changes in the production and emissions of methane into the atmosphere can have dramatic effects on global warming and climate change. Emissions on an annual basis tend to be highly variable with increased production in the Northern hemisphere and during the summer growing months (Steele et al., 1987). Anthropogenic sources of methane (e.g. fossil fuels, landfills, rice cultivation, ruminants, etc.) account for the majority of annual production (ca. 430 Tg methane) and are reflected in the dramatic increase in atmospheric concentrations of methane over the last 200 years (Etheridge et al., 1998; Schlesinger and Bernhardt, 2013). Despite the fact that anthropogenic sources of

methane dominate contemporary emissions, wetlands are the dominant natural source (66.5% of natural sources; Schlesinger and Bernhardt, 2013) or around 20-30% of total methane emissions (Bousquet et al., 2006; Bloom et al., 2010; Ringeval et al., 2010), and it has been suggested that they can account for a large amount of the interannual variability (Bousquet et al., 2006). In total, wetlands may contribute as much as 150 Tg of C as methane on an annual basis, which is estimated to be up to 3% of the overall net ecosystem productivity (NEP) of these systems (Dlugokencky et al., 2011). In addition to the atmospheric efflux, wetlands account for the single largest stock of soil C, accounting for some 50% of the global total (Gorham, 1991; Tarnocai et al., 2009). However, this C is relatively un-decomposed and has potential for rapid turnover and release into the atmosphere. Taken together, estimates of the global C budget must factor in wetlands as both a major sink and source of greenhouse gas emissions, with potential for large fluctuation annually and into the future under altered climate regimes and other anthropogenic stressors.

1.1.3 Controls on Methane Production and Emissions in Peatlands

Carbon is the building block of life on Earth, and organisms have evolved to use C containing molecules as both sources (reduced) and sinks (oxidized) of electrons. Methane, the most "simple" hydrocarbon molecule, is both a product of and reactant for microbial metabolism. Methane emissions from peatlands occur as a balance between activities of methanogens, and methane oxidizing microbes (Lai, 2009). Abiotic factors are generally easier to investigate and scale-up to globally-relevant terms, and a number of factors play a role in the turnover of C in peatlands as well as the production of methane. Current understanding centers on water (anoxic conditions) and temperature as controlling factors, although other variables such as pH and

nutrient availability play critical roles, but these dynamics are complex and are just beginning to be understood (Ulanowski and Branfireun, 2013). Lack of knowledge on methane production controls in peatlands is mainly due to high variability within and among sites as well as temporal dynamics that further complicate general trends for abiotic factors. However, O_2 availability and temperature are more consistent predictors of methane production across broad distributions of northern peatlands (Lupascu et al., 2012). More recently, research is also pointing towards more complex biotic interactions between bacterial fermenters and methanogens in peatlands that can impact methane production (Wüst et al., 2009), and very intriguing gramminoid plant controls on both methanogens (Rooney-Varga et al., 2007) and methanotrophs (Kip et al., 2010). While these biotic controls on methane emissions are driven by plant community composition, dynamics are complex, and unraveling these processes is challenging (Ward et al., 2013). Thus, responses of methanogens and methanotrophs to biotic changes must be framed in the context of broader abiotic changes as well. Other obvious gaps exist in our knowledge that is exemplified by members of the Thaumarchaeota (formerly grouped in the Crenarchaeota) that are common in peatlands (Lin et al., 2012; Basiliko et al., 2013; Hawkins et al., 2014; Bomberg, 2016), and have been indirectly linked to methane oxidation using DNA stable-isotope probing techniques (Gupta et al., 2012), but in general have no clearly defined function. Isolates of the Thaumarchaeota exist (Stieglmeier et al., 2014), however isolates from peatlands have yet to be obtained from any of the Thaumarchaeota, and no isolates from the Paravarchaeota, Aigarcheota, Diapheotrites, Aenigmarchaeota, Woesearcheota, and Nanoarchaeota exist to date.

Hydrology

The two broad classes of peat-forming wetlands, fens and bogs, vary dramatically in their hydrology. Fens typically have a more dynamic water flux, while bogs are relatively stable, even with drying and rewetting events (Deppe et al., 2010). Two scenarios are considered when it comes to water table dynamics and future climatic trends including wetter fens, often the result of permafrost melt at high latitudes, and drier bogs. In laboratory incubations it has been shown that fens have high methane production potential if saturated (Juottonen et al., 2005), and that potential future production could be upwards of 30x greater than in bogs with periodic drying and rewetting episodes (Deppe et al., 2010). Conversely, bogs that dry out and have a lowered water table position, are likely to experience a reduction in methane emissions but will also experience increased overall C and CO_2 loss (Estop-Aragonés and Blodau, 2012). In both peatland types, dynamics are further complicated by variability in microbial community composition with changing water table positions. Using terminal restriction fragment length polymorphism (TRFLP) analysis, Yrjälä et al., (2011) found that water saturation at four different levels in a peatland lead to distinct microbial communities. Additionally, it has been found that at depth, prolonged saturation and water table stability leads to more similar methanogen communities, while surface communities in more variable hydrologic conditions leads to more diverse communities (Kotiaho et al., 2010). The influence of water on methane production often acts in concert with temperature as drying or wetting often also leads to changes in temperature dynamics. While these effects have been considered together in lab incubations or the context of permafrost melt (e.g. Estop-Aragonés & Blodau, 2012; Mackelprang et al., 2011; Negandhi et al., 2013), it is far more common that the variables have been considered separately.

Temperature

As with all biological and chemical reactions, temperature plays a vital role in methanogenesis in peatlands, with rates increasing as temperatures rises due to changing climate or seasonality (Kotsyurbenko et al., 2007; Dinsmore et al., 2013). Globally, *in situ* peatland methanogenesis also exhibits temperature optima that are higher in peats from warmer climates and cooler in northern (arctic/boreal) wetland environments (Bartlett and Harriss, 1993), likely due to changes in the temperature optima of the bacterial populations. In contrast, many methanogens exhibit relatively high temperature optima when in culture, with temperatures around 35-37 °C being optimal for *M. boonei* (Bräuer et al., 2011) but *in situ* optima may be closer to 20-25 °C (Kotsyurbenko et al., 2004). Temperature driven increases in methane production rates are also characterized by changes in microbial community composition with warming decreasing richness and abundance of methanogens (Kim et al., 2012). It is likely that temperature-related changes in community structure are influenced by peat depth and the initial methanogen community composition. However, studies have often only looked at community composition at the end of varied treatments to see how they have changed relative to the presumably same starting point. This is a problem that needs to be methodologically addressed so that changes can be conclusively attributed (or not) to abiotic factors.

Permafrost thaw, while changing the hydrology of peatlands and exposing previously frozen soil organic matter to more biologically favorable temperatures, will also likely cause an initial pulse of methane release due solely to the thaw (Mackelprang et al., 2011; Hodgkins et al., 2014). However after initial pulses, the rates of methane production have not been found to differ significantly between colder arctic and warmer subarctic peatlands with Q_{10} (reaction rate change with 10 °C increase) values nearly identical (Lupascu et al., 2012). Instead it is likely that the

plant communities are driving/mediating the release of methane in peatland systems with *Sphagnum* sites having a higher Q_{10} than sedge sites (global averages of 8 and 4.3, respectively; Lupascu et al., 2012). This poorly understood plant-temperature interaction is further complicated by the buffering capability of methanotroph communities living in oxic surface peat. A recent study conducted by van Winden, et al. (2012) showed that up to 15 °C. *Sphagnum* mosses could effectively mediate methane emissions even with increased bacterial methane oxidation activity, however beyond that point the buffering capacity was exceeded and high levels of methane were released. Although this may only be a problem for more temperate peatlands, it still highlights the potential for interactions between plant community composition and temperature, as well as the broader balance between methanogens and methanotrophs.

Role of Metals and Sulfur

The role of metals and sulfur has also been investigated in regards to methanogens and methane production. Many trace metals are needed in micronutrient quantities for cellular components. So far methanogenesis has been found to be enhanced by additions of Ni, Fe, Co and Mo (Basiliko and Yavitt, 2001; Kida et al., 2001; Hu et al., 2008; Evranos and Demirel, 2015), and negatively impacted by Cu (Mao et al., 2015). Interestingly, acetoclastic methanogens appear to be more tolerant of Cu compared with hydrogenotrophic methanogens (Karri et al., 2006) and an isolate strain (*Methanobacterium bryantii*) has even been found to be tolerant of high Cu concentrations while growing on formate (Kim et al., 1996). Overall, the concentration of the heavy metals is critical and most metals can play a toxic, inhibitory, and stimulatory role depending on methanogen species and bioavailability of the metals (Zayed and Winter, 2000; Mudhoo and Kumar, 2013). Furthermore, most studies have focused on systems with micronutrient

concentrations or additions. Sulfur (S), primarily in the form of sulfate, has been shown to have an inhibitory effect on methanogenesis (Watson and Nedwell, 1998; Granberg et al., 2001; Gauci et al., 2004; Eriksson et al., 2010), likely via competition for H_2 and/or CH_3COOH with sulfate reducing bacteria, which have an energetic advantage over methanogens. Additionally, it has been shown that the reduction in methane production is proportional to peat sulfate concentrations (Yavitt et al., 1987) however an upper bound to this effect is not understood and it appears that at very high concentrations electron donor availability and plant interactions become factors. While initial steps have been taken, it is apparent that there is still a need for a better understanding of the influence sulfur and metals play in the production of methane and methanogen community composition in peatlands.

Methane Oxidation

The oxidation of methane entering the atmosphere occurs primarily in the atmosphere (ca. 95%) where hydroxyl radicals interact with methane in a series of reactions ultimately producing HO_2, H_2O, CO_2, and O_2 following the breakdown of the intermediary products formaldehyde and CO (Cicerone and Oremland, 1988; Neef et al., 2010). While constituting a smaller portion of atmospheric methane oxidation, removal of methane by biological pathways (methanotrophy) is still a key process and in many cases serves as a very important filter prior to release into the atmosphere. Until recently it was thought that methane was only consumed through aerobic oxidation by members of the Proteobacteria, however increasing evidence shows that less known bacterial species from other phyla (e.g. acidophilic *Verrucomicrobia*) might also play a role (Dunfield et al., 2007) and have recently been shown to have a diverse ecosystem and global distribution (Sharp et al., 2014). In addition to aerobic methane production, anaerobic oxidation

of methane has been found to not only occur in peatlands (Smemo and Yavitt, 2007), but is a widespread phenomena; though controls on this process and the organisms involved remain elusive (Gupta et al., 2013). Of the processes proposed it is likely that reverse methanogenesis is a common pathway, using a nickel (Ni) containing F430 cofactor variant and protein similar to the F430 cofactor/metyl-coenzyme M reductase used in methanogenesis (Krüger et al., 2003).

Climate Change

All of these variables and potential controls must be framed in the context of a changing climate, although predictions regarding responses of peatlands and the global C cycle to change are difficult to make due to their broad distribution, interacting effects, and great deal of site- or region- specific variability (Avis et al., 2011). For example in maritime peatlands experiencing increases in brackish water from sea level rise, or in sites receiving industrial deposition, increased SO_4^{2-} and altered electron acceptor availability will likely enhance bulk peat decomposition and suppress methanogenesis (Sutton-Grier et al., 2011). Rising global temperature will be a driving force with variable responses predicted, but overall a net efflux of methane and CO_2 is predicted (Avery Jr et al., 2001; van Winden et al., 2012). Peatlands at high latitudes where permafrost presence is reliant on freezing temperatures are more at risk with relation to increasing temperatures and subsequent methane release. In effect a few degree Celsius change induces a "state change" causing permafrost to thaw (Shaver et al., 1992; McGuire et al., 2010; Koven et al., 2011). This is concerning as 88% of soil C in northern wetlands is in permafrost (Tarnocai et al., 2009) and with loss of permafrost, many wetlands will drain (Avis et al., 2011). Water loss will lower the water table leading to drying of surface peat and oxic conditions increasing losses of C from both decomposition and dissolved organic

carbon (DOC) loss with the increased hydrologic export (Olefeldt and Roulet, 2012). Drainage either directly due to human activity (e.g. peat and mineral mining, road construction, forestry) or indirectly due to climate change not only will cause potential longer-term C loss from decomposition but also makes peatlands targets for wildfires (Mack et al., 2011; Turetsky et al., 2011). Finally, elevated CO_2 is likely to have effects on peatland ecosystems although it is difficult to predict the exact response due to plant communities being the driving force (rhizosphere interactions and NPP shifts) at local scales. Increased root exudation is predicted as photosynthesis is stimulated, increasing DOC losses and methane production (van Groenigen et al., 2011). This is not only a concern in natural peatlands but also in rice paddies, which account for 25% of methane production from wetlands (Wang et al., 2004) and is estimated to increase 43% under projected increases in CO_2 (van Groenigen et al., 2011).

1.1.4 Methanogens

Methanogens are strict anaerobic Archaea in the *Euryarchaeota* phylum that are direct descendants of some of the oldest life on earth (Kral et al., 1998) likely originating ca. 3.5 billion years ago, as indicated by fossil evidence (Ueno et al., 2006). As members of the domain Archaea, methanogens possess a number of distinct physiological traits from bacteria and other higher organisms including, an archaellum for motility and anchoring similar to bacterial type IV pili (Thomas et al., 2001; Orell et al., 2013; Shahapure et al., 2014); phospholipids with an ether-linked glycerol backbone, branched isoprene chains, and even lipid monolayers (Koga and Morii, 2005; Oger and Cario, 2013); and distinctive lack of peptidoglycan cell wall which is replaced with various outer envelopes having diverse composition often containing pseudomurein (Albers and Meyer, 2011). Methanogens are the only known organisms containing

the entire metabolic pathway required for methanogenesis, and it is thought that methanogens and the methanogenesis pathways are surviving remnants; lost in non-methanogens (Gribaldo and Brochier-Armanet, 2006; Borrel et al., 2011). This unique metabolism relies on a limited number of simple organic molecules and/or H_2 for C and redox substrates, commonly products of fermentation produced primarily by anaerobic bacteria.

Historically, methanogens (and most Archaea) were phylogenetically grouped into a "none of the above category" within prokaryotes (Woese and Fox, 1977). More formal classification of methanogens has a relatively recent history and was initiated with the use of the small subunit ribosomal ribonucleic acid (SSU rRNA) gene and the discovery of Archaea by Woese and Fox in 1977 (Balch et al., 1977; Fox et al., 1977; Woese and Fox, 1977) . Since then understanding of methanogen phylogeny has developed rapidly with seven orders of methanogens currently recognized including Methanococcales, Methanopyrales, Methanobacteriales, Methanomicrobiales, Methanocellales, Methanosarcinales, and Methanomassiliicoccales. Methanogenesis has also been predicted among the Bathyarchaeota (Evans et al., 2015). The SSU rRNA gene and the ubiquitous methanogen functional gene *mcrA* (encoding for the *A* subunit of the methyl coenzyme-M reductase) allow us to easily detect methanogens in a range of environments and both generally agree and work well for phylogeny (Luton et al., 2002). However, we are now becoming more dependent on enrichment and culturing techniques for confirmation of genotypes and phenotypes within the methanogens. This trend is partially because physiology, metabolism, and whole genome sequences are used to classify microbial species (Liu, 2010) but also because large gaps exist in the methanogen culture collection with entire clades and orders un- or under-represented (e.g. RCII and *Methanocellales*).

Metabolic Pathways

Methanogenesis is a low energy yield (ΔG°) pathway, and is often the terminal step in anaerobic decomposition; relying on byproducts of fermentation (low MW C compounds and H_2). In a metabolic sense methanogens have little diversity, relying on a limited number of substrates and three primary pathways of methanogenesis differing only in the initial steps of production. By far the most widespread pathway to methane production is via the hydrogenotrophic route (Eq. 1) where electrons from H_2 gas are used to reduce CO_2 (the acetyl–CoA pathway is used to fix CO_2; Deppenmeier et al., 1996). Methylotrophs are the second classification of methanogen metabolism and are mainly in the Methanosarcinales order. These organisms are known to produce methane form a wide variety of methylated chemicals such as methanol and dimethylsulfide (Liu, 2010). Acetoclastic methanogens form the third metabolic grouping and are in effect a subset of methylotrophs, deriving their energy from the methyl group of acetate (Eq. 2). The genus *Methanosaeta* (formerly *Methanothrix*) comprises the only obligate acetrotrophs comprising of only four species (Kamagata et al., 1992; Mori et al., 2012). Other species in the genus *Methanosarcina* have been shown to also grow on acetate, however all of these species also undergo general methylotrophy and some can even undergo all three methanogenesis pathways (Liu, 2010).

Equations

1) $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$

2) $CH_3COOH \rightarrow CO_2 + CH_4$

Distribution

Methanogens are likely all descendants of a single common ancestor, which is strongly supported by their common final steps in metabolism (Deppenmeier et al., 1996) as well as their broad distribution into many diverse and often termed "extreme" niches including varied pH (Bräuer, Yashiro, et al., 2006), temperature (Kamagata and Mikami, 1991), and salinity (Mori et al., 2012). Outwardly only two conditions need to exist for methanogens to be active in an environment: 1) it must be anoxic and 2) there must be chemicals present that offer the opportunity for an energy gain; that is a reaction with a $\Delta G° < 0$. Common habitats include landfills, peatlands, rice paddies, geothermal vents and hot springs, sediments (lake, river, and ocean), and digestive tracts of ungulates, humans, and termites (Chaban et al., 2006). While methanogenesis is an obligately anaerobic process, methanogenic species have been found to inhabit micro-oxic environments including root rhizoshperes (Erkel et al., 2006), oral biofilms (Vianna et al., 2009) and water columns (Bogard et al., 2014). Given the resilience and diversity of methanogens they are truly a globally distributed group of organisms, and speculation even exists that puts methanogens as prime candidates for life outside of Earth (Kral et al., 2004; Webster et al., 2015).

1.2 Research Objectives

My PhD research aims to fundamentally expand the current knowledge of the poorly understood organisms involved in methane cycling in peatlands, and looks to fill crucial gaps in the current understanding of methane production in peatlands and methanogen communities. Specifically, my thesis will i) investigate methane production in peatlands sampled around the world in an effort to better understand if common factors universally control methane production, ii) link microbial communities to methane emissions from a region impacted by historic sulfur and metal

deposition in an effort to better understand the role metals play in methane production on a regional scale, and iii) use enrichment techniques to identify and describe novel methanogens that are important in the production of methane in peatlands ecosystems. Taken together this research looks to understand the role of methanogens and methane production in peatlands in a variety of scales ranging from characterizing global controls on production to discovering novel species.

1.3 Research Approach

In order to better understand the role of methane production in peatlands and the microorganisms responsible for methane production, this research takes a top-down approach, first looking at global patterns and controls on methane emissions, then looking at more specific local factors controlling methanogen communities and methane emissions across the regions surrounding the former largest global point source of S, Ni, and Cu emissions, and finally looking at methanogens themselves in an attempt to establish a more comprehensive view of methane production in peatlands. In Chapter 2 a set of globally distributed samples from 105 distinct peatland sites were collected and analyzed using a standardized method making the methane production values directly comparable. This is the first time in vitro methane production has been looked at in this way and will lead to broad conclusions regarding the global and universal controls of methane production in peatlands. In Chapter 3, I use a regional gradient of sulfur and metal deposition to investigate the roles metals and sulfur play in methane production and methanogen community composition, allowing us to draw broader conclusions of the role metals and sulfur in methane production at other scales or under future anthropogenic impacts. Finally in Chapter 4, I use enrichment and culturing techniques as well as DNA sequencing technologies

to identify novel methanogens that are currently unknown and uncultured. This furthers our understanding of metabolic constraints on methanogens and provides us with novel sequences that can be added to the underrepresented library currently used to identify methanogens in ecosystems. It also uniquely highlights the ecological linkages between these novel methanogens and their bacterial syntrophic partners. Taken together my thesis research approach highlights the importance of multi-scale approaches, particularly in the field of environmental microbiology that is inherently methods-limited and focuses on micron-scale targets (Madsen, 2015), and emphasizes the importance of conducting research and considering results in multiple contexts.

1.4 Significance of Research

This thesis represents an important contribution our understanding of methane production in peatlands and microbial communities involved in regulating methane emissions. Many aspects of this research are novel in nature including the first global analysis of methane production, use of a globally unique (severe) pollution gradient that includes highly impacted sites to elucidate controls on methanogen communities and methane production, and the incorporation of enrichment work aimed at specific "high impact" organisms that to date remain uncultured. This thesis explains global and regional controls on methanogen communities and methane production and expands our knowledge of methanogen diversity, increasing our current knowledge and understanding of methane dynamics in peatlands on multiple scales.

1.5 Thesis Structure

1.5.1 Chapter 1 Introduction

This chapter provides general background information and a literature review, providing context for the interaction of peatlands and resident methanogens. Current gaps in knowledge are addressed and sets the stage for the subsequent research chapters. The research objectives, approaches, and significance are also discussed providing the logic behind the organization of thesis as a whole.

1.5.2 Chapter 2 Patterns and predictors of methane production across peatlands globally

The first experimental chapter is focused on characterizing methane production across a set of globally distributed peatlands. This is part of a larger project looking at the microbial communities and serves to set the stage for the thesis by looking at methane production in peatlands in a global context; attempting to link commonly measured variables and/or specific site characteristics to methane production.

1.5.3 Chapter 3 Altered Methanogen Communities and Methane Production in Northern Peatlands Following Long-term Smelter Deposition of Ni, Cu, and S

Chapter 3 determines the role S and metals play in methane production and methanogen community composition in a region heavily impacted by smelting. By providing a higher resolution study in a smaller geographic region, this chapter looks to answer more specific questions regarding methane production and methanogen community composition.

1.5.4 Chapter 4 Environmental enrichments for novel methanogens: Approaches for obtaining uncultured organisms in the age of rapid sequencing

The final research chapter had a primary goal of discovering and describing new methanogens. Conceptually this is the finest resolution and was undertaken to address the need for expanding the methanogen reference library to interpret single-gene-based microbial fingerprinting approaches. Without this basic research, fingerprinting studies of methanogen communities will continue to suffer and struggle to make sense of data, as sequences usually do not match to known methanogens.

1.5.5 Chapter 5 Conclusions

Chapter 5 is a summary of the three research chapters and serves to link the thesis together and draw broader conclusions. Additionally, this chapter offers suggestions for improvements in this topic area and further research directions to consider in the future.

Chapter 2 Patterns and predictors of methane production across peatlands globally

2.1 Abstract

Methane is a potent greenhouse gas and while peatlands cover little global surface area, their soils produce a disproportionately large amount of methane. While *in situ* field measurements of methane production are ideal, they are often not practical and labs commonly employ other methods to estimate potential methane production for a given site. A standard measurement protocol is the laboratory incubation, where a small sample of peat is incubated in a closed container under controlled conditions. However, individual labs conduct these experiments in modified forms making direct comparison of potential production across and sometimes within sites impossible, greatly hindering our ability to conduct a global meta-analysis of potential global methane production. This study looked to overcome methodological shortcomings by obtaining and incubating peat samples from globally distributed peatlands using standardized conditions and a common methodology. In total 423 samples were incubated from all seven continents and 105 distinct sites over an 80 day period, with methane measurements taken at regular intervals. Measures of temperature, pH, water table position, latitude and longitude, total elements, structural chemistry (FTIR), and von post as well as broad plant communities were used to determine if there is a "universal control" or predictor of methane production in peatlands on a global scale. Methane production is increased with peat chemistry low in acids and aromatics, and high in carbohydrates as well as in sites with more neutral pH and *Carex* and more broadly the Cyperaceae plant communities present. Five percent of samples had a

disproportionately high production of methane compared to CO_2, highlighting the important role methane plays in peatland systems in regards to GHG production. Our study highlights the importance of a common methodology in soil science and indicates that peatland type, pH, and vegetation are the likely drivers of broad scale potential methane production.

2.2 Introduction

Peatlands are major stores of soil carbon (C), but are likely to release large amounts of methane in the coming decades due to their high vulnerability to changing climates, especially in high northern latitudes (IPCC and Working Group I Technical Support Unit, 2013). Global estimates indicate that peatlands store an estimated 1/3-1/2 of the global soil carbon while only occupying ca. 3% of land area, and are currently considered to be a global carbon sink (Gorham, 1991; Lehner and Döll, 2004; Schlesinger and Bernhardt, 2013). However peatlands are also estimated to emit between 5-10% of annual atmospheric methane, a greenhouse gas (GHG) more potent than CO_2 (Schlesinger and Bernhardt, 2013).The net contribution of GHG emissions is a balance of production and consumption in the system (Juottonen et al., 2012). Production by methanogenic archaea depends on bacterial fermentation of root exudates and other complex C substrates to produce low molecular weight C substrates (e.g. CO_2 and acetate) and H_2 (Cadillo-Quiroz et al., 2010; Xu et al., 2012), which is both produced and consumed by bacteria (Liu, 2010). Microbial communities also mitigate net methane release through consumption via both anaerobic and aerobic methanotrophy (Wüst et al., 2009). Therefore, although the net methane emissions are due to in large part to the balance of microbial production and consumption (Lai, 2009), these can both vary in both space and time due in large part to abiotic and biotic influences. For example, it is thought that methane production is largely controlled by

temperature (Bubier, 1995; Bergman et al., 2000), vegetation, and water table position and fluctuations (Kotiaho et al., 2010; Moore et al., 2011; Yrjälä et al., 2011; Lupascu et al., 2012). Plant communities may facilitate methane release through increased C substrates in the rhizosphere (Chanton et al., 1995; Joabsson et al., 1999; Ström et al., 2003; Hodgkins et al., 2014) and plant litter (Valentine et al., 1994; Verhoeven and Toth, 1995; Yavitt et al., 2000) but also through physical processes such as transport through aerenchyma cells and altered peat structure affecting ebullition, effectively bypassing zones of methanotrophy (Leppälä et al., 2011; Klapstein et al., 2014).

The production and emission of methane from peatlands has been investigated in many contexts that estimate net emissions *in situ* (e.g. eddy flux and static chambers; (Eriksson et al., 2010; Nadeau et al., 2013). However, these methods make it difficult to establish the controls on methane production as they measure the balance of production and consumption in a given system (net emission). One of the most common and fundamental research methods for better estimating methane production is measuring potential methane production in laboratory incubations. This method is often used as a part of a larger study and gives a reasonable estimate for the potential a site has to produce methane under a given set of conditions. The most recent analysis of global potential methane production in peatlands was done in 1998 by Segers, who synthesized together results from individual studies concluding that ecosystem type, temperature, aeration, location (latitude), and water table (WT) position were only weakly associated with methane production. Furthermore, he found that anaerobic C mineralization was a major control on methane production in that large amounts of CO_2 are used for reducing electron acceptors making it unavailable for methanogenesis. These conclusions have framed much of the

subsequent work and it is apparent that a contemporary look into potential methane production is needed. Peatlands are highly variable within sites and at broad spatial distribution making responses to the global C cycle difficult to predict (Andersen et al., 2013; Bridgham et al., 2013). Furthermore, whether peatlands are influenced by common factors are still poorly understood, in part due to the fact that most studies consider only local conditions or use different methodologies for measuring production rates that are not always directly comparable across studies (Yavitt et al., 1987; Segers, 1998; Lupascu et al., 2012). Importantly care must be taken to avoid common pitfalls of literature reviews such as positive publication bias (Coursol and Wagner, 1986; Callaham et al., 1998; Sarewitz, 2012) and direct comparison of data obtained using varied methodologies (Turetsky et al., 2014).

The objective of this study was to characterize methane production and related controls in an unprecedented global peatland sampling effort. We evaluated a set of global peat samples for methane production using a common method (i.e. so *in vitro* rates were comparable across all sites) and then related commonly measured peatland characteristics including *in situ* temperature, pH, plant community, and water table position as well as factors including location (longitude, latitude, elevation), peat humification, and organic-structural and elemental- chemistry to determine if a single factor or combination of factors can be used to better predict the potential for peatland methane production on a global scale. Here we seek to answer: (1) what abiotic measurements are directly correlated to methane production across a range of global samples, (2) is there a model of combined abiotic factors that best predicts global methane production (3) can broad plant community composition predict methane production in peatlands, and (4) are there

sites that have a disproportionately high contribution of methane or CO_2 and if so what unique characteristics might be leading to these high production rates.

2.3 Methods

2.3.1 GPMP Methodology

The samples used in this study are part of a larger project looking at microbial communities in peatlands across the globe. Individual research groups recruited into the study were asked to follow the same set of standardized sampling procedures. At each site, researchers collected samples from three centralized locations from the predominant topographic position, representative of the site. For sites with existing experimental manipulations, participants were instructed to collect one core from each replicated plot or up to three cores for each treatment depending on the specific study design. Different methods could be used for core collection (e.g. Russian style corer, box corer, sampling by gloved hand with a serrated knife) but four depth increments (0-10 cm, 10-20 cm, 30-40 cm and 60-70 cm) were obtained from each coring location, avoiding compaction and cleaning tools between sampling with ideally at least 50 g field moist peat per depth. Immediately upon sub sampling depth horizons, temperature, von Post humification, and soil moisture scores were recorded. Surface samples were stored in a plastic bag (e.g. whirl-pak) and lower depth increments were homogenized in a plastic bag with a representative sample placed in a sterile 50 ml Falcon tube for DNA analysis. The remaining materials for all downstream applications were kept in plastic bags and these, as well as samples for DNA extraction and analysis were frozen to -20 °C prior to shipping. Collaborators used rapid shipping methods, and when possible samples were shipped on dry ice or ice packs to

ensure samples remained frozen. Upon arrival at the USFS lab (Houghton, MI), samples were catalogued and stored at -20 °C. Ancillary data were recorded including, the sampling date, sampler (personnel), site and plot name, core and depth codes, plant community composition (% cover of 1 m^2 quadrat), species richness, a photograph, maximum vegetation height, large vegetation (tree and shrub) characteristics within a 5m radius of sampling, latitude, longitude, elevation, pore water pH, depth to WT, size of peatland, and any other pertinent information the researchers could provide.

2.3.2 Gas Production

For this work only, the 30-40 cm depth horizon was used for measuring anaerobic methane and CO_2 production, as this depth was commonly (82.5% of sites) under the water table but still shallow enough to have a substantial effect on methane emissions to the atmosphere *in situ*. Sub samples of the frozen peat were obtained in person from the USFS lab by breaking or using a serrated knife to cut a 5-10 g wet weight piece of frozen peat from the homogenized peat sample. Samples were then transported on ice to Laurentian University (Sudbury, ON) and were again stored at -20 °C. Prior to incubations, samples were allowed to thaw gradually over three days at 6 °C. Methane and CO_2 production was then measured for 423 peat samples from October 2015 to June 2016 in four groupings of 90-120 samples as follows. For each sample, between 5 and 30 g of field moist peat (depending on sample quantity available and density) was weighed into a 125 ml glass-canning jar. Twenty milliliters of deionized and degassed water were added covering the peat, and jars were capped with lids containing integrated rubber septa. Samples were then made anaerobic by replacing the headspace gas with nitrogen. Jars were attached to a vacuum manifold and the headspace was first removed then replaced with ultra-high purity N_2.

This process was repeated a total of four times and the headspace gas was allowed to come to ambient pressure. Anaerobic jars were incubated in a BioChambers SPC-56 growth chamber (Winnipeg, MB) set at 15 °C in the dark without shaking. Headspace methane and CO_2 production was measured on the following days of incubation: 5, 10, 15, 20, 30, 40, and 80.

On each sampling date, 5 ml of N_2 gas was injected into each jar and thoroughly mixed into the headspace. A 5 ml gas sample was then removed and run on a gas chromatograph (GC) fitted with a 1 ml sample loop, 3 m packed column tailored for gas separation, a methanizer jet to reduce CO_2 to methane, and a flame ionization detector (SRI 8610C, Torrance, CA). The oven temperature was set at 105 °C and each sample took three minutes to run. Sample peak areas integrated by the PeakSimple data acquisition and GC controller software were compared to a 10 ppm commercial standard gas, which was run every 30 samples as a check. Less regularly additional standards were prepared by dilution of a pure methane sample to ensure that the peak area response was linear across a 1-10,000 ppm range, and thus that a single point calibration was adequate. Final values ($\mu g\ C \cdot g^{-1}$ dry peat $\cdot\ day^{-1}$) were calculated by first converting to ppm then to μg C as either CO_2 or methane using the ideal gas law and headspace volume of individual jars. Values were then adjusted for mass of dry peat and converted to rates using incubation time. Running totals were calculated as the mass of C measured on a given day plus the sum of mass removed (as CH_4-C or CO_2-C) from previous sampling dates.

2.3.3 Peat and Water Properties

The peat pH was measured on day 80 following the final gas measurement. An additional 20 ml of DI water was added and samples were measured using an Accumet AB150 while being

continually stirred with a magnetic stir bar. Peat was then dried at 60 °C until constant weight was obtained to determine dry weight and initial water content. Finally, the peat was ground to <1 mm using a Wiley style mill for archiving.

2.3.4 Chemical Analysis

For the elemental analysis of samples a subset of the original peat was oven dried at 60 °C and ground to a fine powder in a ball mill. One portion was then sent to the Chanton Lab at Florida State University for Fourier transform infrared spectroscopy (FTIR) analysis while the other portion was sent to Laurentian University for preparation for combustion-based (CNS) elemental analysis, loss on ignition (LOI), acid digestion and elemental analysis by inductively coupled plasma mass spectrometry (ICP-MS). Loss on ignition was determined by combusting a sample of dry ground peat at 550 °C for 6 hours in a muffle furnace (shorter protocols left visible char in the sample). Prior to combustion, peat was weighed to 0.0001 g on a digital balance into a 10 ml ceramic crucible, and an initial peat mass of ca. 0.1 - 0.5 g was weighed (with efforts to use as close to 0.5 g as possible when enough peat was available). Following mass loss, samples were again weighed and the proportion of mass lost was calculated at the LOI %. Ash was transferred to a digestion tube using type I grade purified water for digestion and ICP-MS analysis. Ashed peat samples were subjected to an acid digest and total elemental analysis on an ICP-MS at the Elliott Lake Field Research Station at Laurentian University (Abedin et al., 2012; Watkinson et al., 2017). First peat ash in water was digested twice at 110 °C for 210 minutes in 10 ml of HF and HCl until dry. Then 7.5 ml of HNO_3 acid and HCl was added at 110 °C for 250 minutes for the third digest until dry. A final digest consisting of 10 ml of HNO_3, 2 ml of HCl, and 0.5 ml of HF was carried out at 110 °C for 60 minutes. Approximately 8 ml of liquid remained and

samples were diluted to a total of 50 ml using deionized water. Prior to analysis on a Varian 810 ICP-MS, samples were diluted again to conform to the instruments detection limits. Duplicate samples and standard reference materials were included every 20 samples to ensure data quality.

2.3.5 Carbon, Nitrogen, and Sulfur

For CNS analysis dried ground peat was weighed to 0.0001 on a digital balance. Approximately 75 mg of peat was placed into a formed tin foil capsule along with 150 mg of tungsten (VI) oxide powder. The tin foil was then folded over sealing the sample and was compressed in a pill-shaping device. Packed samples were then shipped to the Watmough Lab at Trent University for analysis on an Elementar VarioMacro CNS Analyzer and precision of results was confirmed using blanks and sulfadiazine for CNS recalibration and QA standards (NIST-1515-SRM).

2.3.6 FTIR

Fourier transform infrared spectroscopy (peat organic C quality) was performed following methods of Hodgkins et al. (2014). Ground peat samples forcibly held in place on an ATR crystal using a PerkinElmer Spectrum 100 FTIR spectrometer fitted with a CsI beam splitter and a deuterated triglycine sulfate detector. Spectra between wavenumbers 4000-650 cm^{-1} and % transmittance were both recorded from the average of four scans. Peaks locations were corrected individually to account for peak shifts due to sample chemistry variation and a baseline correction was applied. After corrections, spectra were converted from transmittance to absorbance. Measured spectra were grouped by peak heights at specific wavelengths into "acids" (carboxylic acid: 1,720 cm^{-1}; Niemeyer et al., 1992; Haberhauer et al., 1998; Cocozza et al., 2003; Gondar et al., 2005), "carbohydrates" (polysaccharides: 1,030–1,080 cm^{-1}; Grube et al., 2006)), "aromatic" (lignins/phenolic: 1,513–1,515 cm^{-1} and aromatics:1,600–1,650 cm^{-1};

Niemeyer et al., 1992; Cocozza et al., 2003), "aliphatics28" (aliphatics: 2,850 cm^{-1}; Niemeyer et al., 1992), and "aliphatics29" (aliphatics: 2,920 cm^{-1}; Niemeyer et al., 1992).

2.3.7 Statistical Analysis

All statistical tests and figures were produced using R version 3.3.1 (R Core Team, 2016). Raw data were compiled from individual labs and combined and pre-processed using the tidyr and dplyer packages (Wickham and Francois, 2016; Wickham, 2017). Missing values were replaced with NAs and for chemical analysis where samples were below the detection limits values were considered NA as interpolation and method detection limit replacement were ineffective in downstream analyses. Due to missing data in various sections the actual sample number evaluated (n) is reported when appropriate (figures and tables). Summary statistics were calculated using the stat.desc() function from the pastecs package (Grosjean and Ibanez, 2014). Prior to correlation and regression analyses, data were first tested for normality and transformed appropriately. Correlations (Pearson's) were conducted using functions from the Hmisc package (Harrell, 2016) and a Bonferroni correction was applied to p-values. Regressions were preformed using the MASS package (Venables and Ripley, 2002) and stepwise regressions were conducted using "both" directions (forward-backward). Three multiple regression models were used to account for the large number of missing data in structural chemistry and elemental chemistry analysis, optimizing the number of samples that entered each model. Log transformed methane production data were used in all regression and correlation models. In order to evaluate if methane was major contributing factor to GHG production relative to the contribution of CO_2 equivalents were calculated by multiplying CH_4-C values by a factor of 25 (Shindell et al., 2009) prior to dividing by the CO_2-C value, giving the CO_2 equivalent ratio. The site map was

generated using the ggmap package (Kahle and Wickham, 2013) and associated GPS coordinates for each site location.

2.4 Results

2.4.1 Global Summary

In total there were samples from 18 countries from all seven continents comprising of 105 unique site locations (Figure 2.1). In total, 423 were incubated and included in the overall study. The global mean methane emission over the 80 day incubation is presented in Table 2.1 and was 111 (μg CH_4-C/g dry peat / day) with a minimum value of 0.01 from the "Victor mine" site in Ontario, Canada (Lat 52.697263, Lon -83.891776) and maximum production of 1,822 in the "Pastoruri" site in Peru (Lat -9.884810, Lon -77.188270). Daily production rates of CO_2 and total C mineralization (measured as the sum of CO_2 and methane) were 1055 and 1166 (μg C· g^{-1} dry peat · day^{-1}), respectively (Table 2.1). Core temperature at the time of sampling ranged from frozen samples in sites with continuous or discontinuous permafrost to a maximum of 31.4 °C in the tropical Indonesian sites. Global pH average was 4.9 and percent moisture 86.4% (Table 2.1). The water table was positioned on average 17.8 cm below the peat surface. In total, 66 samples were above the water table at the time of sampling, while 311 of the incubated samples were below the WT at the time of sampling (46 samples were missing WT data). Additional details for values of interest for the global dataset are reported in Table 2.1.

2.4.2 Global Controls on Methane Production

Values for global mean plant percent cover are presented in Table 2.1. *Sphagnum* and Ericaceae were dominant in many sites with *Carex* and Cyperaceae being less common. Methane

production was significantly ($p<0.05$) higher in sites where *Sphagnum* and Ericaceae were absent, with a decrease of 2x and 1.9x, respectively (Figure 2.2). In contrast, the presence of *Carex* and Cyperaceae species tended to increase the overall methane production with sites containing *Carex* producing 65% more methane than sites that lacked *Carex*. Similarly, sites containing Cyperaceae had a significant increase in methane production by 53% over sites without Cyperaceae present.

A number of factors were found to correlate with methane production potential across the global dataset on an individual basis (Table 2.2). Methane production was significantly ($p<0.05$) correlated with pH ($r = 0.24$), moisture ($r = 0.33$), and structural chemistry elements including carbohydrates ($r = 0.25$), aromatics ($r = -0.32$), and acids ($r = -0.41$). Of particular note is pH which was significantly correlated to most measured variables excluding temperature, moisture, CO_2 production, and aromatic peat structural chemistry (Table 2.2). Panels A and B in Figure 2.3 show the positive relationship of peat pH (post incubation) and peat moisture with methane, respectively. P-values in both instances were <0.001 and indicate that an increase in moisture and pH both lead to an increase in methane production. Finally a number of variables initially expected to influence methane production had no significant relationship, including organic matter (LOI and percent C), and S and Ni contents. CO_2 production, a proxy for overall microbial activity, shared many common correlates to methane (Table 2.2) including a positive correlation with peat moisture ($r = 0.45$, $p<0.001$) and conversely a negative, but insignificant, correlation to pH ($r = -0.18$). Other relationships between individual factors are shown in Table 2.2.

The structural components of the peat samples were evaluated using FTIR and three of the components (carbohydrates, aromatics, acids) correlated significantly ($p<0.05$) with the log of methane production. Of these, acids (carboxylic group) and aromatics (primarily lignin and phenolic compounds) were both strongly and negatively related with methane production (Figure 2.3 C and D, respectively). In contrast to the other structural components, carbohydrates (Figure 2.3 E) was positively related with methane production ($r^2=0.25$, $p< 0.001$). Aliphatic28 and aliphatic were both negatively correlated with methane production but less than other structural components (Table 2.2) and were highly correlated ($r^2= 0.99$) with each other. The humification index (HI) is a good substitute for direct humification measures and tends to increase with decomposition in peat can be calculated as the ratio of aromatics, aliphatic28, and aliphatic29 to carboxylic peaks (Broder et al., 2012). These ratios were calculated and tested against methane production but no significant correlations were seen; p=0.27, 0.19, and 0.21, respectively.

2.4.3 Multiple Regression Models for Predicting Methane Production

Model 1 (Table 2.3) is primarily composed of variables commonly measured in peatland studies that require little special equipment to obtain. The final model omitted CO_2 production, percent S and N and retained pH, moisture, LOI, percent C and the ratio of C to N. The resolved model had a p-value < 0.001 and an R^2 of 0.31. In the second model (Table 2.4) the structural organic chemical components measured by FTIR were added to the first model along with peat temperature at time of collection. This model was better at predicting methane production with an $R^2= 0.42$ and included CO_2, carbohydrates and acids in place of C and C:N from the first model. Finally a third model was tested (Table 2.5), which included the elements Co, Fe, Ni, P in addition to the variables in model 2. This third model explained a similar amount of methane

production (R^2= 0.40) and retained the same structure as the second model while replacing LOI with total Ni. The number of observations in the three models decreased substantially from 393 observations in the first model to 257 in the second and 183 in the third. This is in part due to sample availability for chemical analyses but also a large number of samples that were below detection limits for elemental analysis.

2.4.4 Disproportionately High Methane Production

Greenhouse gas production in terms of CO_2 equivalents (greenhouse warming potential; GWP) are shown in Figure 2.4. Twenty samples (right of red line) contribute disproportionately high amounts of GHG emissions as methane (Figure 2.4). The rest of the samples are centered on zero indicating that in most sites there is a normal distribution of GHG production and the role CO_2 and methane play are comparable in terms of GWP within the anaerobic zone. Overall GWP must be considered in the context of the whole peat profile and it is likely that the contribution of CO_2 *in situ* is higher than values represented here. Both *Sphagnum* and Ericaceae are lower in high production samples and *Carex* and Cyperaceae are higher in their sites (Table 2.6), consistent with presence absence data from the global data set (Figure 2.2). In comparisons between individual variables, major differences occurred primarily in pH and acid peak height from the FTIR analysis (Figure 2.5). The mean value for pH was increased by 1.54 units in the high producers, and acid peak height was reduced by a factor of 4.12x compared to the global averages.

2.5 Discussion

In this study we measured methane (and CO_2) production in peat soils under standardized conditions from an unprecedented global sampling effort to explore global scale controls on C mineralization as methane in peatlands. The global average temperature in the peat was 13.9 °C, which substantiates our choice in incubation temperature of 15 °C. We found a significant but weak negative correlation between temperature at time of sampling and potential methane production, indicating that colder peats may be more active when warmed, compared with warm peats that are cooled down. Importantly this means that there should not only be concern for rapid release during initial permafrost melting (Koven et al., 2011) but also continued loss at higher than average peat temperatures in historically cool climates. There is often a link between temperature (and seasonality) and the water table position in peatlands where temperature is at a maximum in mid summer when water table is lowest (Peichl et al., 2014). We found the global water table position at an average depth of 17.8 cm below the peat surface and confirming that the 30-40 cm depth used for our incubation was saturated and anoxic in the majority of our samples. This also means that there is a substantial aerobic zone in peatlands that is on average around 10-20 cm deep across the globe. This aerobic zone plays an important role in mitigating net release of methane into the atmosphere via methanotrophy (Dunfield et al., 2007; Smemo and Yavitt, 2007; Gupta et al., 2013; Sharp et al., 2014), thus the methane production we measured in our incubations likely overestimate emissions compared to *in situ* values.

There is often a strong link between plant functional types and pH in peatlands where more neutral sites tend to have less *Sphagnum* cover and more *Carex* species present (Strack et al.,

2006). In this study we showed that there are links between plant community structure in terms of both presence/absence as well as abundance that indicate sites with higher *Sphagnum* and Ericaceae cover tend to produce less methane. This is consistent with other studies that have found individual sites dominated by *Sphagnum* have lower average methane production than sites either lacking *Sphagnum* (Rooney-Varga et al., 2007; Juutinen et al., 2010; Ward et al., 2013) or dominated by other species (Strack et al., 2017). Often coincidental with *Sphagnum* presence is a decrease in pH, which is a known driver of microbial communities in peatlands (Godin et al., 2012; Myers et al., 2012) as well as most other soil environments (Fierer and Jackson, 2006). Plant communities are also a driving factor in microbial community structure (Andersen et al., 2013; Jassey et al., 2013; Robroek et al., 2015) and while we didn't measure the microbial community directly, other work supports the idea that there are likely different microbial (both bacterial and methanogen) communities along the global pH gradient, potentially contributing to the difference in potential methane production. This is further supported by work that has shown higher rates of methane production in peatlands with higher pH (typically rich fens) compared to peatlands on the poor fen to bog spectrum that tend to have a lower pH and less methane production (Valentine et al., 1994; Kotsyurbenko et al., 2007). Taken together, our work indicates that sites typically categorized as rich fens likely have more potential for methane production than sites that are both more acidic and dominated by *Sphagnum*.

When considering all predictive variables together our results show that there are a number of reasonable explanatory models for potential methane production. Other studies have found that pH (Ye et al., 2012), moisture (Ma et al., 2012; Dinsmore et al., 2013), and peat composition (Niemeyer et al., 1992; Hodgkins et al., 2014) have major roles in production of methane.

Similarly, we found that pH and moisture were the best predictors appearing in all three models. Overall microbial activity measured as CO_2 production only enters these models when peat structural chemistry is also included indicating that there may be a link to carbon quality that is important in overall microbial activity and methane production. In our final model, total Ni was retained. This metal is important as a central element forming a tetrapyrrole in the F430 cofactor only found in methanogens and anaerobic methanotrophic archaea and plays an essential role in the methyl coenzyme M reductase (*mcrA*) the terminal enzyme catalyzing methane production (Scheller et al., 2010). While this result is interesting and shows a direct link to methane production, it was absent in all other correlations and regression models so interpretation is difficult. It is clear though that there is still a large amount of work needed to determine the ecological concentration limitations of Ni and the role it plays in peatlands and methane production (Zayed and Winter, 2000; Basiliko and Yavitt, 2001; Mudhoo and Kumar, 2013; Evranos and Demirel, 2015). Other elements known to be related to methane production (Co, Cu, or Fe: Mo was below detection limits and not evaluated) were not found to be important in the multiple regression models, however Co was independently correlated with methane production, and deserves further attention, as there has been little work looking at its role as a trace nutrient affecting methane production in soils (Basiliko and Yavitt, 2001; Glass and Orphan, 2012).

Finally, we found that there were sites that had a disproportionately high contribution of GHG production in the form of methane. In general the conclusions drawn from these 20 samples is reflective of the dataset as a whole and indicated that pH, plant community, and peat structural elements are major factors in a disproportionately high methane production. It is interesting to consider that aside from these 20 samples there was a relatively normal distribution of methane

and CO_2 meaning that while methane may be important in some "hot spot" locations within peatlands in general *in vitro* incubations suggest a comparable contribution of GHG from CO_2 and methane . Of these 20 samples two locations had numerous samples including "Pastoruri", "Cahvish", and "Quileayhuanea" near Catac, Peru (3, 2, and 1 samples, respectively) and "Saline" and "Poplar" fens near Fort McMurray, Alberta (Canada; 2 and 5, respectively). Collectively these four sites were spatially close (ca. 10 km radius within each country) and accounted for 13 of the 20 high producer samples. Other high producers were from geographically distinct locations and were likely a random occurrence of high production within the sites. Together there is evidence that indicates that the Peru and Alberta locations may have unique site/regional characteristics that are leading to high methane production; although it is unclear from the given data what these may be. Additionally, the large number of normally distributed sites globally suggests that the role CO_2 production plays in peatlands could be being underestimated in many peatland studies. There is often a link between methane and CO_2 production and labile C compounds (Valentine et al., 1994; Chanton et al., 1995) where increased rates of CO_2 indicate more microbial activity in general and often times more methane as the result of increase substrates for methanogenesis in the forms of low molecular weight organic C or CO_2 directly (Hodgkins et al., 2014). However, we found no correlation between the two gasses on a global scale. Studies that have found direct relationships between CO_2 and methane are still valid at a local scale (Moore and Dalva, 1997), but it is likely that CO_2 is serving as a sort of proxy for other variables such as peat quality (humification) or root exudates that are less variable within more fine scale spatial distribution.

2.6 Conclusions

In conclusion, this work is the first of its kind that has incubated a wide range of peat from across the globe under standardized conditions allowing for direct comparisons in potential methane production rates and controls. We found that abiotic factors including moisture, and nutrient concentrations (e.g. Ni, N, P) can influence potential methane production, however, linkages were tenuous. More substantial were the links between methane production and pH as well as the plant communities and overall peat structural chemistry (FTIR). Here we saw that peatlands with higher pH and plant communities containing *Carex* and Cyperaceae (often coincidental) often had the highest potential methane production, and defined most of the sites that had disproportionately high methane production. Peat structural chemistry containing increased levels of acids, aromatics, and carbohydrates tended to produce more methane and these are likely driven by plant community structure. According to our results, CO_2 production and overall microbial activity was not a good predictor of methanogen activity on a global scale. Future studies will likely continue to occur at the local and regional scales, but care should be taken to use make data as relatable to other regions as possible. Adapting a method from standard practices because it is logical for a specific location may be okay for comparison within a site, but makes extrapolation to other areas difficult and we would discourage this practice for future incubation work.

2.7 Tables and Figures

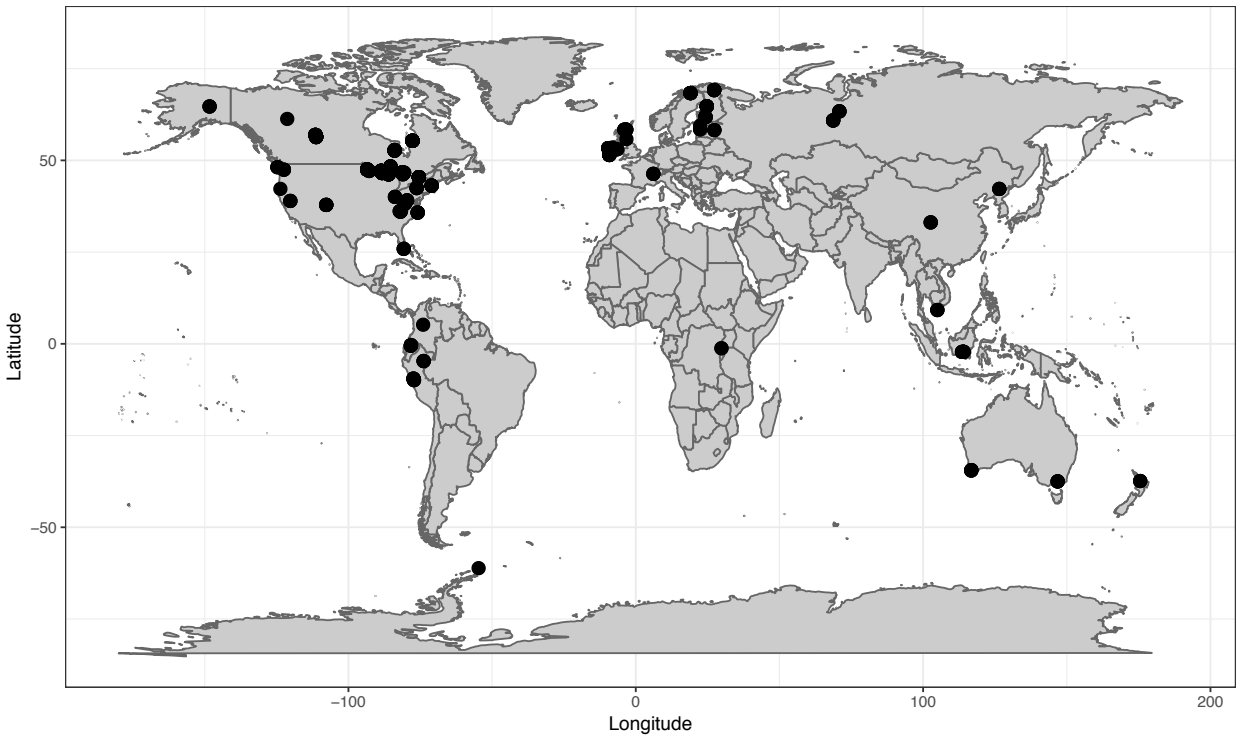

Figure 2.1 Sample site map. Locations of 105 sites where peat was obtained for methane incubations.

Table 2.1 Summary of global dataset with mean, median, minimum, and maximum values. The number (n) of samples reflects the number of complete observations for a given variable.

	Total	
Continents	7	
Countries	18 (1)	Australia, Canada, China, Colombia, Ecuador, Estonia, Finland, Indonesia, Ireland, New Zealand, Peru, Russia, Scotland, Sweden, Switzerland, Uganda, USA, Vietnam, (Antarctica)
Sites	105	
Samples	423	

Measurement	Mean	95% CI	Median	Minimum	Maximum	n
Methane (μg/g dry/ day)	111	22.4	28	0.01	1822	423
Carbon Dioxide (μg/g dry/ day)	1055	69.6	909	23.7	4882	423
Total C mineralization (μg/g dry/ day)	1166	74.2	1006	23.7	4893	423
Core Temperature (°C)	14	0.5	14	Frozen	31	344
pH (incubation)	5	0.1	5	3.4	8	423
Moisture % (incubation)	86	1.0	1	22.7	97	423
Depth to Water Table (cm)	18	2.3	13	-50.0	91	356
Carbon %	42	1.2	45	1.7	80	395
Nitrogen %	1	0.1	1	0.1	5	395
Sulfur %	0	0.1	0	0.0	6	395
***Sphagnum* %**	42	3.9	32	0.0	110	406
***Ericaceae* %**	41	8.0	15	0.0	525	400
***Carex* %**	13	2.1	5	0.0	126	392
***Cyperaceae* %**	19	2.8	5	0.0	126	363

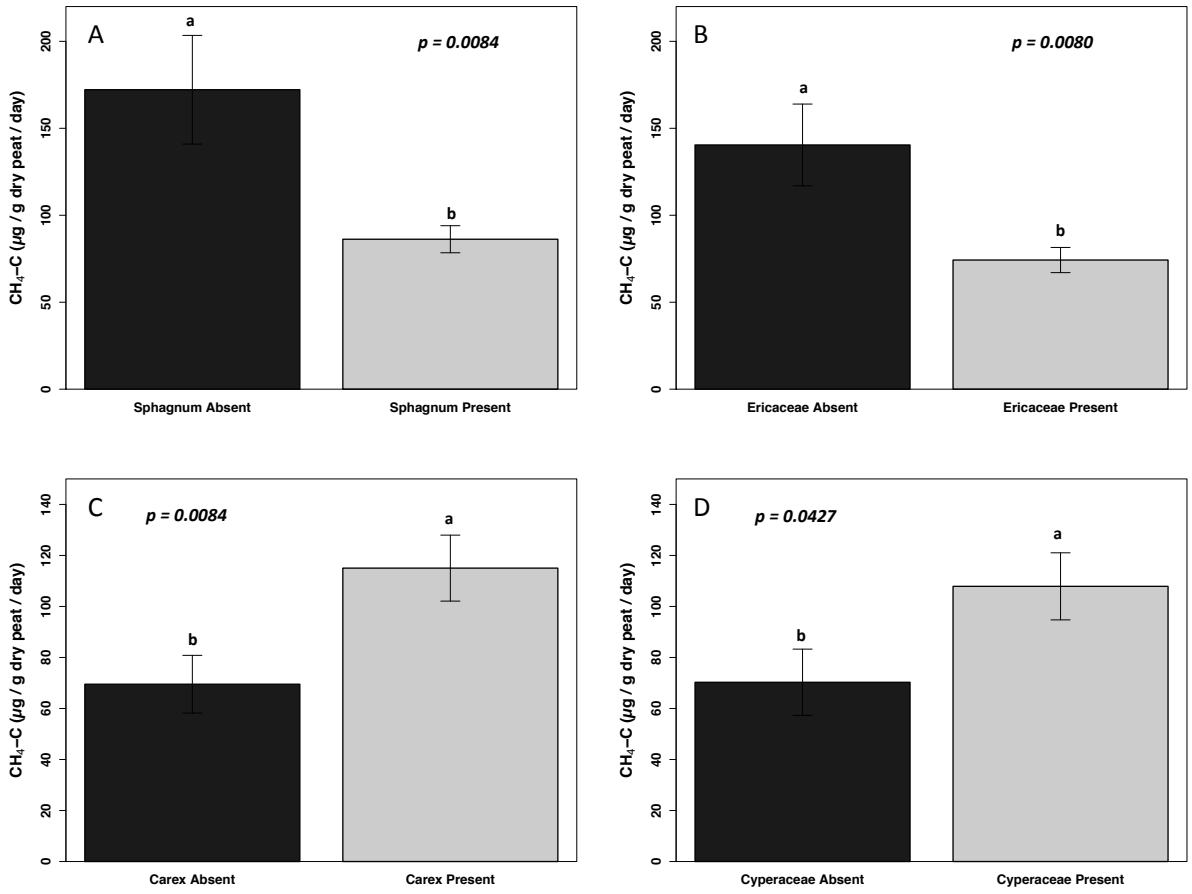

Figure 2.2 Total methane production over an 80-day incubation by plant groupings based on presence/absence data for *Sphagnum* (A), Ericaceae (B), *Carex* (C), and Cyperaceae (D). The number of samples (n) present for each grouping (*Sphagnum*, Ericaceae, *Carex*, and Cyperaceae) were; present: 271, 270, 237, 229; absent: 136, 123, 155,134; and no data reported: 16, 25, 31, 60, respectively.

Table 2.2 Pearson's correlation (r) of field and lab measurements related to methane production. Bold and italicized values are significant with a p<0.05 after a Bonferroni correction.

	Methane	Core Temperature	pH	Moisture	Carbon Dioxide	Carbohydrates	Aromatics	Acids	Aliphatics-28	Aliphatics-29	LOI	N %	C %	S %	C:N	Total Co	Total N
Methane	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Core Temperature	-0.12	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
pH	***0.24***	-0.14	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Moisture	***0.33***	0.09	-0.27	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Carbon Dioxide	0.12	-0.01	-0.18	***0.45***	--	--	--	--	--	--	--	--	--	--	--	--	--
Carbohydrates	***0.25***	***-0.43***	***-0.29***	***0.30***	***0.33***	--	--	--	--	--	--	--	--	--	--	--	--
Aromatics	***-0.32***	***0.37***	-0.20	-0.17	-0.21	***-0.38***	--	--	--	--	--	--	--	--	--	--	--
Acids	***-0.41***	***0.34***	***-0.78***	0.01	0.02	0.05	***0.42***	--	--	--	--	--	--	--	--	--	--
Aliphatics-28	-0.16	0.11	***-0.21***	-0.17	-0.18	-0.13	***0.52***	***0.27***	--	--	--	--	--	--	--	--	--
Aliphatics-29	-0.15	0.10	***-0.21***	-0.17	-0.19	-0.12	***0.53***	***0.27***	***0.99***	--	--	--	--	--	--	--	--
LOI	0.03	0.05	***-0.48***	***0.66***	***0.43***	***0.30***	0.17	***0.36***	0.09	0.10	--	--	--	--	--	--	--
N %	0.12	0.00	***0.25***	0.15	0.01	***-0.32***	0.19	***-0.39***	***0.33***	***0.33***	0.09	--	--	--	--	--	--
C %	-0.05	***0.21***	***-0.44***	***0.66***	***0.31***	-0.19	***0.60***	***0.53***	***0.40***	***0.41***	***0.80***	***0.31***	--	--	--	--	--
S %	0.02	-0.01	***0.22***	0.00	-0.10	-0.17	0.18	-0.09	***0.25***	***0.26***	-0.09	***0.23***	0.01	--	--	--	--
C:N	-0.11	0.00	***-0.37***	0.32	0.19	***0.24***	-0.03	***0.42***	***-0.29***	***-0.28***	***0.44***	***-0.64***	***0.33***	***-0.22***	--	--	--
Total Co	0.13	-0.19	***0.57***	***-0.40***	***-0.25***	-0.16	-0.19	***-0.53***	-0.08	-0.08	***-0.57***	0.09	***-0.54***	***0.32***	**-0.43**	--	--
Total Ni	0.07	-0.16	***0.37***	***-0.40***	***-0.37***	-0.21	-0.05	***-0.32***	0.06	0.06	***-0.58***	0.06	***-0.50***	***0.23***	**-0.44**	**0.76**	--
Total P	0.17	***-0.29***	***0.34***	-0.17	-0.12	-0.12	-0.07	***-0.44***	-0.04	-0.03	***-0.32***	0.16	***-0.25***	0.09	**-0.28**	**0.57**	**0.45**

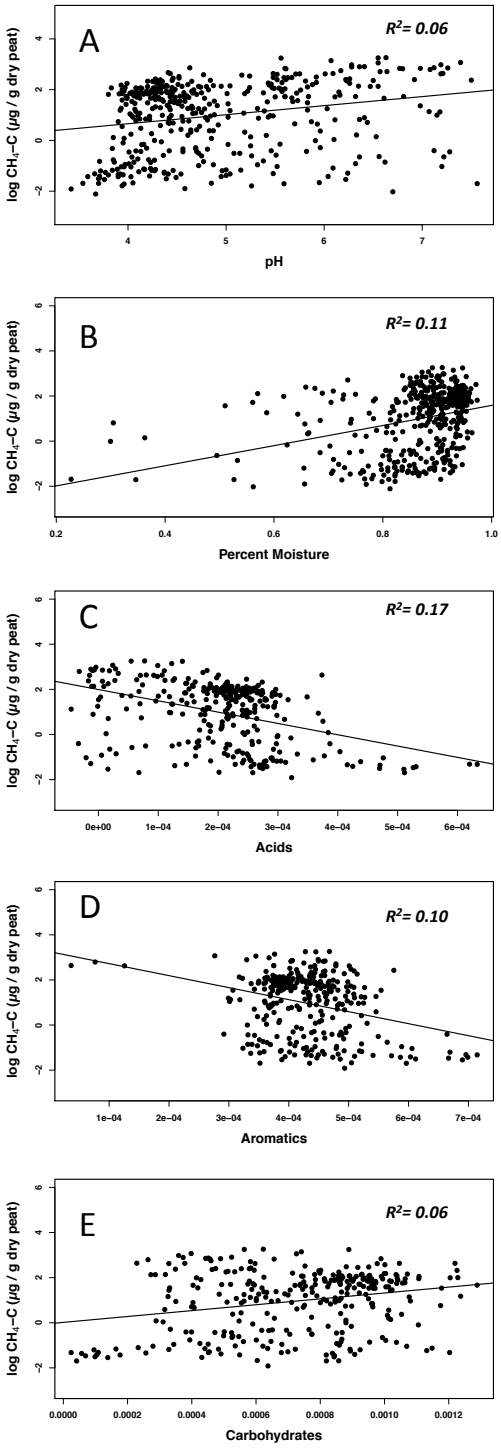

Figure 2.3 Methane simple regressions. Comparing total methane production (log transformed) with pH (A), percent moisture (B), Acids (C; FTIR), Aromatics (D; FTIR), and Carbohydrates (E; FTIR) of the most individually correlated variables. All five regressions were significant at a p<0.001 and had N values of 423, 423, 308, 308 and 308, respectively.

Table 2.3 Stepwise regression comparing methane production to a variety of baseline predictor variables commonly measured in peatland studies. AIC model selection criteria were used and a forward-backward progression for individual steps. The overall model contained 393 complete observations.

Model 1

Input model:

Methane ~ pH + Moisture + CO_2 + LOI + N + C + S + C:N

Resolved model:

Methane ~ pH + Moisture + LOI + C + C:N

	Estimate	p-value
Intercept	-6.099	< 2e-16 ***
pH	0.410	< 2e-16 ***
Moisture	7.897	4.45e-08 ***
LOI	0.661	0.07832 .
C	-0.047	1.50e-07 ***
C:N	-0.009	0.00561 **

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Multiple R-squared	0.31
Adjusted R-squared	0.30

F-statistic: 34.53 on 5 and 387 DF, p-value: < 2.2e-16

Table 2.4 Stepwise regression comparing methane production to a variety of baseline predictor variables in addition to peat structural C measures and percent C, N, and S. AIC model selection criteria were used and a forward-backward progression for individual steps. The overall model contained 257 complete observations.

Model 2

Input model:

Methane ~ Temperature + pH + Moisture + CO_2 + Carbohydrates + Aromatic + Acids + Aliphatics28 + Aliphatics29 + LOI + N + C + S + C:N

Resolved model:

Methane ~ pH + Moisture + CO_2 + Carbohydrates + Acids + LOI

	Estimate	p-value
Intercept	-7.90	8.85e-10 ***
pH	0.34	0.00530 **
Moisture	8.54	2.62e-12 ***
CO_2	-0.0002	0.04580 *
Carbohydrates	1408	3.32e-06 ***
Acids	-2907	0.00201 **
LOI	-0.69	0.13636

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Multiple R-squared	0.42
Adjusted R-squared	0.41

F-statistic: 31.83 on 6 and 260 DF, p-value: < 2.2e-16

Table 2.5 Stepwise regression comparing methane production to a variety of baseline predictor variables in addition to peat structural C measures and percent C, N, and S as well as total elemental data for elements known to influence methane production. AIC model selection criteria were used and a forward-backward progression for individual steps. The overall model contained 183 complete observations.

Model 3

Input model:

Methane ~ Temperature + pH + Moisture + CO_2 + Carbohydrates + Aromatic + Acids + Aliphatics28 + Aliphatics29 + LOI + N + C + S + Total Co + Total Fe + Total Ni + Total P + C:N

Resolved model:

Methane ~ pH + Moisture + CO2 + Carbohydrates + Acids + Total Ni

	Estimate	p-value
Intercept	-7.81	1.63e-08 ***
pH	0.24	0.058899 .
Moisture	8.32	2.61e-11 ***
CO_2	-0.00017	0.118493
Carbohydrates	1372.0	0.000107 ***
Acids	-3338.0	0.001748 **
Total Ni	0.33	0.008078 **

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Multiple R-squared	0.40
Adjusted R-squared	0.38

F-statistic: 20.74 on 6 and 185 DF, p-value: < 2.2e-16

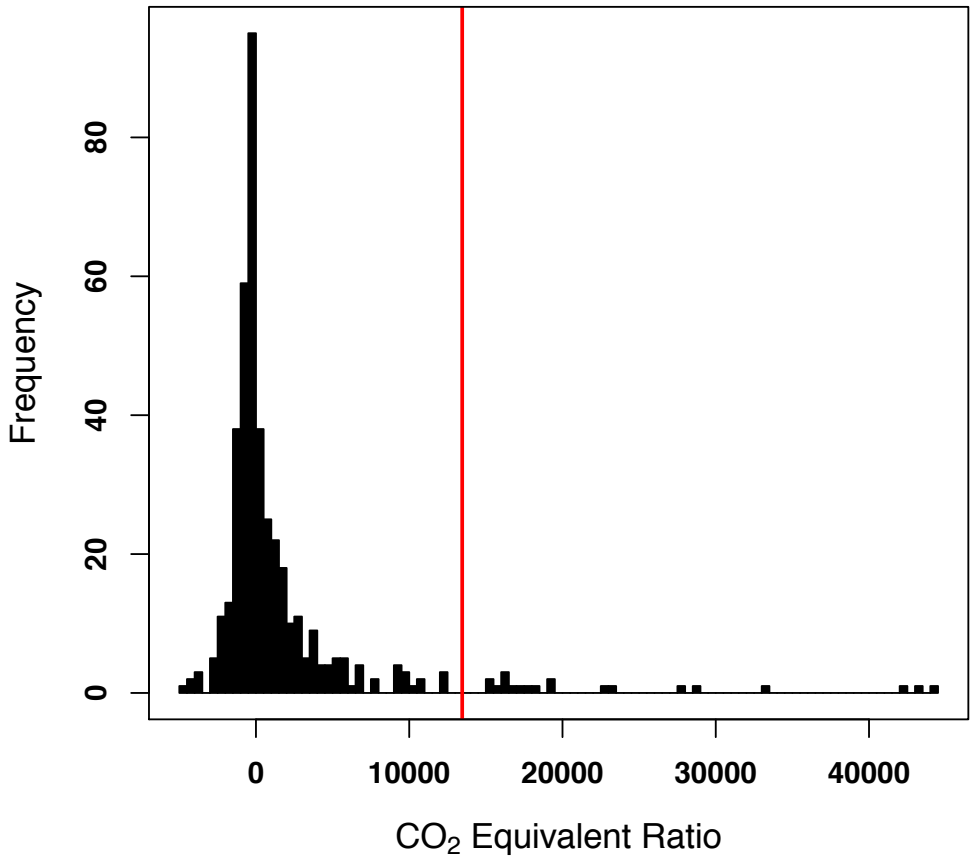

Figure 2.4 Relative contribution of methane and CO_2 to GHG production on a per carbon mass where methane was multiplied by a factor of 25x. Values to the left of zero had a larger contribution from CO_2 while values to the right of zero were dominated by methane production. Value equal to zero had an equal contribution from both gasses. The red line is 2 SD from the mean and the 20 samples the right had a disproportionately high contribution of GHG derived from methane.

Table 2.6 Vegetation cover for the full dataset of samples compared to samples that had a disproportionality high contribution of GHG coming from methane.

	Full Data			High Producers			t-test (welch)		
	% Cover (mean)	se	N	% Cover (mean)	se	N	p value	t	df
Sphagnum	42.02	1.98	406	11.95	6.20	20	0.0001	-4.623	23.09
Ericaceae	41.20	4.06	400	9.21	4.99	14	<0.0001	-4.968	35.38
Carex	12.65	1.07	392	28.36	8.72	14	0.0965	1.7876	13.395
Cyperaceae	19.48	1.44	363	40.00	10.35	14	0.0705	1.9637	13.511

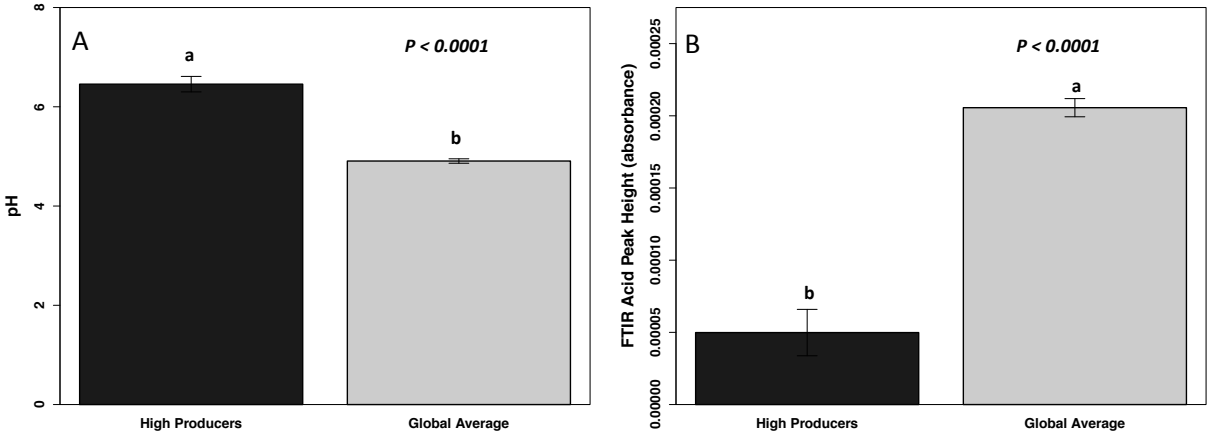

Figure 2.5 pH and Acid mean values (+/- se) for disproportionately high producing methanogens compared to the global average. pH is presented in panel (A) where means were calculated from a total of 20 and 423 samples for the high producers and global average, respectively. Acid peak height measured by FTIR is shown in panel (B) and means are based on n=16 for high producers and n=308 for acid peak height.

Chapter 3 Altered Methanogen Communities and Methane Production in Northern Peatlands Following Long-term Smelter Deposition of Ni, Cu, and S

3.1 Abstract

Peatlands cover a disproportionately large amount of land area in Canada and represent an immense global store of biospheric carbon and freshwater, serving critical ecosystem functions and often linking terrestrial and aquatic systems. Few studies have looked at the role metals and sulfur play in methane production in peatlands. Sudbury, ON (Canada) offers a unique study system with a history of metal smelting (primarily Ni and Cu) and subsequent metal and sulfate deposition that has accumulated in regional wetland soils. This research targeted ten peatlands across an established pollution gradient to determine how methane production and the methanogen communities have responded to chronic high metal and sulfur input. Data indicate that proximity to smelters plays a key role in methane production, methanogen abundance (*mcrA* copy numbers), and methanogen community composition (MiSeq of *mcrA*). Metal and sulfur concentrations decreased, while methanogen abundance and methane production increased, with distance from smelters and depth in peat profile. Additionally, methanogen community composition (order-level OTUs) ranges from ca. 15% unclassified in reference sites to ca. 90% unclassified in highly impacted sites, indicating prevalence of unique/poorly described taxa in these communities. Of the OTUs identified in impacted sites, acetoclastic and methylotrophic orders were proportionally higher than hydrogenotrophic methanogen orders that dominated less-impacted sites. Taken together these data indicate that metal and sulfur deposition has

significantly altered methanogen community composition, abundance, and methane production with implications for global climate change and the understanding of methanogen phylogeny.

3.2 Introduction

The increase in anthropogenic atmospheric greenhouse gasses is of increasing concern and peatlands are of particular interest due to their disproportionately large impacts on atmospheric methane and carbon dioxide (CO_2) concentrations. Peatlands are expected to undergo dramatic changes under future climatic conditions and the outcomes of this perturbation on their biogeochemical functioning and feedbacks are still unclear. Peatlands are characterized by high levels of partially decomposed organic material and long-term storage of carbon, due in large part to their anoxic conditions. While these anaerobic conditions limit overall organic matter mineralization, anaerobic pathways continue nonetheless and the production and emission of methane is often the dominant contributor to atmospheric warming from these systems. As a greenhouse gas, methane is estimated to be 25-35 times as potent as CO_2 due to differences in radiative forcing over a 100 year time frame (Lashof and Ahuja, 1990; Shindell et al., 2009; IPCC and Working Group I Technical Support Unit, 2013). Globally, northern peatlands (346 million hectares; Gorham, 1991) account for ca. 3% land area and contribute up to 10% of annual global net methane emissions to the atmosphere (Bridgham et al., 2013; Schlesinger and Bernhardt, 2013). Peatlands also account for between 30-50% of global soil C storage (Gorham, 1991; Tarnocai et al., 2009) . While it is agreed upon that at broad scales methane production from peatlands will increase in the coming years, the specific controls of methane production at local and regional scales are still poorly understood, largely due to site variability and local factors (Moore et al., 2011).

There is a well-established set of abiotic and biotic controls on methane production that currently exist. These are known to include temperature (Kotsyurbenko et al., 2007; Dinsmore et al., 2013), moisture and water table position (Kotiaho et al., 2010), microbial community (Wüst et al., 2009; Kip et al., 2010; Kotiaho et al., 2010), and vegetation (Ward et al., 2013). Peatland plant composition can vary widely both within and among peatlands, greatly affecting methane production and release. Graminoids are known to enhance overall methane production (Robroek et al., 2015; Strack et al., 2017) while mosses likely mitigate methane release under oxic conditions (Strack et al., 2017). Most importantly it appears that plant activity and active net ecosystem productivity is the strongest link to methane production (Waddington et al., 1996; Klapstein et al., 2014), as higher rates of methanogenesis are observed with an increase in labile soil C (Joabsson and Christensen, 2001). However, plant effects are complicated by other factors such as water table position: for example, sites dominated by gramminoid species have more influence on methane release in dry sites compared to wet ones (Waddington et al., 1996; Strack et al., 2017).

Methane emission also increases with higher water table or more saturated conditions (Deppe et al., 2010) and/or with decreased aerobic consumption via methane oxidation pathways (Kip et al., 2010; Gupta et al., 2012). Water table fluctuations can also influence nutrient availability and bacterial communities, in turn impacting the methanogen community composition and activity (Yrjälä et al., 2011). The ideal temperature for methanogenesis is likely around 37 ºC for most species (Bräuer et al., 2004), and peatlands with higher soil temperatures tend to produce more methane (Bartlett and Harriss, 1993; Kotsyurbenko et al., 2007; Dinsmore et al., 2013). The

microbial communities responsible for methane release are controlled by two main groups; the methanogens (producers) and methane oxidizers / methanotrophs (consumers). Acetoclastic methanogenesis, conducted by the Methanosarcinaceae and Methanosaetaceae, represents up to 60% of the methane emissions in efficient systems (Zinder, 1993), whereas hydrogenotrophic methanogenesis (CO_2 reduction is coupled to H_2 oxidation), carried out by all other orders of methanogens, becomes more dominant where decomposition is incomplete (Conrad et al., 2010), as is true for peatlands.

Due to historically high emission rates of Ni and SO_2, among other metals (Freedman and Hutchinson, 1980; Gunn, 1995), Sudbury, Ontario offers a unique study system with a history of metal smelting (primarily Ni and Cu) and subsequent metal and S deposition (via H_2SO_4 greatly increasing H^+ ions in the region as well), which have accumulated in regional lakes, soils, and wetland systems. For example, in a 2001 soil survey the Falconbridge area recorded Ni levels in the top 5 cm as high as 3,700 mg/kg. Further, mean values for the region (263.2 mg/kg) are still far above the provincial standard of 150 mg/kg. A historic and contemporary gradient exists for metals and S with decreases in concentration with increasing distance from the smelters and effects of deposition have been studied in terms of vegetation shifts (Gignac and Beckett, 1986) as well as water, peat chemistry, and coarse-scale bacterial and fungal fingerprinting (Luke et al., 2015). Recent research has also looked at metal concentrations in Sudbury sites with respect to plant communities and surface concentrations of metals, finding that metal concentrations in peat are still high and plant diversity and richness is negatively impacted (Barrett and Watmough, 2015). However, the response of the methanogen community responsible for methane production has yet to be researched.

The goal of this study was to use a metal and S deposition gradient to elucidate the long-term effects and difference in methane production and methanogen community composition. More specifically we hypothesized four changes regarding site chemistry, methane production, and methanogen communities; 1) methane production will be lower in impacted sites due to shifts in site chemistry (e.g. due to high S), vegetation, and methanogen communities, 2) a distinct shift in methanogen communities will occur in impacted sites with lower diversity, abundance, and with an increase in methanogens with adaptive metabolisms endemic to polluted sites, 3) deeper peat will be less affected by smelting activities and will have less variation in pH, site chemistry, methane production, and methanogen communities across the pollution gradient.

3.3 Methods

3.3.1 Sampling and Site Descriptions

Ten poor fens located within 60 km of Sudbury, ON were selected along a distance gradient from multiple historic and current smelters similar to past studies of base metal smelter impacts on vegetation and wetland geochemical properties (Gignac and Beckett, 1986; Luke et al., 2015). The region is characterized by shallow granite bedrock overlain by a heterogeneous landscape of lakes, upland barren-lands, white birch (*Betula papyrifera*), replanted pine forests, and peatlands in basins or bridging upland and lake ecosystems. Historically, peatlands in the region are thought to be dominated by *Sphagnum* as evidenced by regional peatlands and low base cation concentrations in surface and ground waters, but by the 1970's after over 90 years of smelting activity nearly all signs of *Sphagnum* mosses were absent from peatlands nearest to the S and

metal point sources and the most-impacted uplands existed in a state of deforestation, except for some stunted white birch (*Betula papyrifera*). This period was followed by a reduction in emissions (SO_2 and metals) from smelters beginning in the 1970's and an intensive land reclamation effort including liming and replanting of upland sites, which have begun to establish forests (Gunn, 1995). In general, sites closer in proximity to the smelters had little to no *Sphagnum* cover with ericaceous shrubs commonly present in high abundance and peat that was more humified (von Post of 7-10); more specific site characteristics are provided in Table 3.1. Prevailing weather patterns cause wind to travel to the East-Northeast and the two sites near Cartier, Ontario located to the Northwest were considered as local controls or reference sites (Figure 3.1), still containing well defined hummocks and hollows, a continuous *Sphagnum* layer, and more acidic and substantially less humified surface peat, in contrast to the more contaminated sites. The surface peat pH and plant communities of the impacted sites selected did not meet the general criteria of poor fens. However this is likely due to the elimination of peat forming *Sphagnum* mosses (that are intolerant of heavy metal deposition) from past pollution deposition (Luke et al., 2015). Based on visible *Sphagnum* peat macrofossils deeper in the soil horizons in most of the sites, it is assumed that these sites were once more like the reference sites prior to disturbance (i.e. with lower pH and a continuous *Sphagnum* understory)

Sampling was conducted over the course of four days in early June 2014. At each site, triplicate cores were taken using a Russian style corer to a depth of one meter below the water table approximately every 20 m along a 60 m transect in the center to reduce edge effect influences (i.e. dust and salt from roads and surface erosion from upland soils as well as natural laggs around the peat soil deposits). Two depth horizons (30-40 cm "middle" and 75-100 cm "deep",

below water table) were selected to investigate variability in methanogen communities and differences in peat chemistry. At each site, samples were collected using a Russian core barrel or by hand with a serrated knife and measuring stick when peat was too fibric for obtaining a reliable core sample. The corer and knife were wiped and washed with deionized water between each sample and cleaned thoroughly with ethanol between sites. Surface pore water was sampled adjacent to each core location. Peat and water samples were stored in new Ziploc® freezer bags and transported on ice. Subsamples were stored at 6 °C for up to a week prior to microbial activity analyses and at -20 °C for DNA-based and chemical analyses.

3.3.2 Peat and Water Chemistry

Peat samples for determination of total elemental concentrations were oven dried at 65 °C, ground in a Wiley mill, and then digested and characterized at the Elliott Lake Field Research Station at Laurentian University. Briefly, 0.5 g of material was mixed in a 9:1 ratio (10 ml) of HF and HCl and digested two times at 110 °C for 210 minutes until dry. A third digestion with 7.5 ml of Nitric acid and HCl was then done at 110 °C for 250 min until dry, followed by a fourth and final digestion with 0.5 ml of HF, 2 ml of HCl, and 10 ml of nitric acid at 110 °C for 60 minutes, leaving 8-9 ml of liquid. Samples were then diluted with ultrapure DI water to 50 ml and stored. Prior to chemical analysis a ten-fold dilution was performed. Extractable elements were obtained from air-dried (25 °C) unground peat samples. Approximately 1 g (dry weight) of peat was extracted by shaking with 40 ml of 0.01M $LiNO_3$ for 24 hours before filtering to 0.45 μm (Abedin et al., 2012). Prior to filtering, the pH of surface pore water and $LiNO_3$ peat slurries was measured directly using an Accument AB-150 pH meter. Both the acid digests and $LiNO_3$ extracts were run on an ICP-MS (Varian 810) set up to detect 38 elements. Duplicate samples

and quality control standards were run every 10 samples to validate data quality and all data were blank-corrected (i.e. trace amounts of elements detected in blank extracts and digests were subtracted from each sample).

3.3.3 Microbial Gas Production

Anaerobic methane and CO_2 production rates were measured for each sample core and at both depths (n=6 per site) on days 2, 4, 8, 16, 30, and 45 of the incubation. Canning jars (225 ml) were filled with ca. 40 g of field moist peat and 30 ml of degassed de-ionized water to ensure anaerobic conditions. Lids fitted with rubber septa were secured and a vacuum manifold was used to flush the headspace four times by first evacuating (helping degas soil water), and then filling the headspace with N_2. Samples were incubated at 20 °C in the dark without shaking. Headspace gases were sampled for methane and CO_2 concentration measurement by injecting 10 ml of N_2 in to each jar (to maintain headspace pressure from subsequent sampling) and mixing the headspace with three syringe pumps prior to removing a 10 ml sample. Headspace gas was injected into a SRI 8610C gas chromatograph (Torrance, CA) fitted with a 1 ml sample loop and a column temperature of 105 °C. Both gasses were measured as methane on a flame ionization detector, with CO_2 being converted to methane by a methanizer jet after passing through the separation column. Peak areas were first converted to ppm using a reference gas standard and then to μg C as methane or CO_2 per gram of dry peat per day using the ideal gas law, headspace volume, peat dry weight, and incubation time. Values on a given day represent the measured value plus the sum of all CH_4-C of CO_2-C removed in previous samplings, giving total production values through time.

3.3.4 DNA Extraction and PCR Amplification

Duplicate DNA extractions for each depth and core were done using the Power Soil™ DNA kit (MoBio, USA), with the manufacturer's protocol modified to include 0.5 g of peat in the bead tube and three washes with 500 µl of 5.5M guanidine thiocyanate (humic acid removal; per Basiliko et al. (2013) prior to the addition of the C5 solution. DNA from pooled replicate extractions was quantified on a H1MG microplate reader (BioTek, VT, USA) with readings at 260 nm ranging from ca. 2-60 ng/µ and $A_{260/280}$ ratios near 1.6-2.0. Two primer sets were used for polymerase chain reaction (PCR) amplification. The 16S rRNA Euryarchaeota specific 1AF(5'-TCY GKT TGA TCC YGS CRG AG-3') – 1100R (5'-TGG GTC TCG CTC GTTG-3') pair (Hales et al., 1996) and a broad-spectrum methanogen-specific functional gene (*mcrA*) set mlasF-mod (5'- GGY GGT GTM GGD TTC ACM CAR TA-3') – mcrAR (5'-CGT TCA TBG CGT AGT TVG GRT AGT-3') (Luton et al., 2002; Juottonen et al., 2006; Angel et al., 2012). PCR mixtures contained final concentrations of the following: 1x Taq buffer (25 mM KCl, 10 mM $(NH_4)_2SO_4$ 0.08% (v/v) Nonidet® P40, and 0.01% (v/v) Tween 20 buffered to a pH of 8.8 with Tris-HCl), 4 mM $MgCl_2$, 1.25 U *Taq* DNA Polymerase (Thermo Scientific), 0.3 µg bovine serum albumin (BSA), 75 pM of each primer, 0.2 mM dNTP mix, 2 µl template DNA (per 50 µl reaction) and sterile DI H_2O to volume. Amplification conditions were optimal using the manufacturers protocol with annealing temperatures of 56 °C for the 1AF-1100R pairing and 55 °C for the mlasF-mcrAR primer pairing. Prior to downstream analyses, both 16S rRNA gene and *mcrA* PCRs were conducted to confirm presence of methanogens. In both cases, PCR products were verified by electrophoresis in a 1% agarose gel.

3.3.5 Sequencing (*mcrA)*

Illumina miSeq for the methanogen functional gene *mcrA* was done on homogenized peat from replicate cores (n=3) for each depth and site using the mlasF-mcrAR primer set. Forward and reverse reads were aligned and quality filtered as described by (Dowd, Callaway, et al., 2008; Dowd, Sun, et al., 2008) and a full fasta and quality file was supplied by the DNA sequencing facility (Molecular Research LP, Shallowater, TX, USA). Data were then processed in Ontario's Compute Canada network (www.sharcnet.ca) using both QIIME and USEARCH8 commands (Edgar, 2010). Barcodes and primers were stripped from reads and the library split into individual samples. Sequences were dereplicated, abundance sorted, singletons discarded, operational taxonomic units (OTU) clustered (97, 90, 85%), chimeric sequences filtered, and OTUs mapped. The *mcrA* gene gives a high-resolution phylogeny and a similarity of ca. 85% has been suggested for "species" level taxonomic classification based on amino acid sequences (Hunger et al., 2011) as well as a classification comparison of *mcrA* and 16S rRNA genes using pyrosequencing sequencing technology (Yang et al., 2014). This yields a taxonomic classification equivalent to ca. 97% for 16S rRNA gene-based phylogenetic analysis. This was conceptually confirmed with all sequences clustering into one OTU for the only cultured representative from a peatland (*Methanoregula boonei*), whereas multiple OTUs were generated for this species when cutoffs of 97% and 90% were instead used for clustering. All representative sequences for OTUs were subsequently checked to ensure they were protein coding using Framebot (Wang et al., 2013).

3.3.6 Quantitative PCR

Quantitative PCR (qPCR) was done in triplicate on DNA from each core for both depths for the *mcrA* gene. Prior to qPCR, both annealing temperature and primer (mlas/mcrArev listed above) concentrations were optimized. A standard was generated from a PCR of a homogenized sample of all DNA extracts (Thermo F126L). The band was cut from the gel and purified (Thermo K069). A ten-fold serial dilution was then made and the 10^{-1}:10^{-5} ranges were run in duplicate for both the middle and deep sample 96 well plates. Each reaction volume was 10µl with 1µl (middle) and 2µl (deep) of sample DNA, 250pM for each primer, 5µl of iTaq™ Universal SYBR® Green Supermix (BioRad 10041157), and water. Reactions were carried out on an Agilent Mx3005P qPCR system. Conditions consisted of 95 °C for 5 min, followed by 45 cycles of 95 °C for 15 sec, 56 °C for 30 sec, and 72 °C for 30 sec. A melting curve was run from 50° to 95 °C to confirm purity of product and indicated no primer-dimer formation. Additionally, homogenized triplicate qPCR product was run on a 1% agarose gel to confirm purity. Reaction efficiencies were 93.1% and 94.7% and had an $r^2 > 0.992$ for both runs. The ASM1 sample was run on both plates for comparison and results were expressed as relative abundance per gram of dry peat normalized to extraction yield (Park and Crowley, 2005). Values ≤3 CT difference from the no template control (NTC) was deemed no different than the NTC and was assigned a value of zero.

3.3.7 Phylogenetic Tree Construction

A phylogenetic tree containing all of the identified 34 OTUs was constructed. Initially a tree was assembled containing one cultured representative from each methanogen family and the OTUs. Unnecessary or redundant cultured representatives were then removed while maintaining tree

branch structure, and sequences of closely related organisms detected in other environmental samples (primarily clones) were added for OTUs that had a large distance to a cultured representative. For each iteration of the tree, sequences were aligned with MAFFT version 7 (Katoh and Standley, 2013), and gaps edited in MEGA (Tamura et al., 2007). Trees were then constructed using IQ-TREE (Nguyen et al., 2015; Trifinopoulos et al., 2016; Hoang et al., 2017) with Bayesian model and 1000 replicates in bootstrap calculations before final edits were done in FigTree (Rambaut, 2014).

3.3.8 Statistics

Statistical analyses of data were done using R version 3.3.1 (R Core Team, 2016). Data were imported and tested for normality (Shapiro test) and transformed appropriately. Summary statistics were conducted using the pastec package (Grosjean and Ibanez, 2014) and comparisons of each variable were done using one-way and two-way ANOVAs for depth, site, and the interaction term using the stats package. When appropriate a post hoc Tukey's HSD test was performed. Principle components analysis (PCA) of site chemistry and canonical correspondence analysis (CCA) of microbial community structure were conducted using the vegan package (Oksanen et al., 2016). For the PCA all chemistry variables were initially included and a representative PCA was subsequently produced with ecologically relevant and significant contributing variables. Sequencing outputs were imported to R using the phyloseq (McMurdie and Holmes, 2013) and biom package (McMurdie, 2014). An indicator species analysis was done using the indicspecies package and groupings included site, depth and the interaction term (De Caceres and Legendre, 2009). Stepwise (forward-backward) regression for methane production

potential was done using the MASS package (Venables and Ripley, 2002) and included the first three principle components from the full PCA analysis.

3.4 Results

3.4.1 Site Characteristics and Chemistry

Sites ranged in distance from 6.8 - 50.2 km from the centroid coordinates (Table 3.1 and Figure 3.1) of the three smelter locations. Dominant plant communities were predominantly *Chamaedaphne calyculata* (leather leaf) in impacted sites and *Sphagnum spp.* in the reference sites (Cartier forest and lawn; CF and CL) with a few exceptions (Table 3.1). Mean values for total and extractable elemental data by site and site*depth are presented in SI Tables 1-4 and were calculated in cases where at least two samples from each grouping were available. In general, the concentration of all metals decreased with distance from smelting activities as well as in depth through the peat profile. Extractable (bioavailable) element concentrations were consistently lower than total elements (typically less than 1%). Peat pH as well as pore water pH (data not show) decreased with distance from smelting activities (Table 3.1) from 5.1 to 3.5. Additionally, von Post measures indicated that peat was more humified in contaminated sites compared with undisturbed ones and in deeper peats compared to surface samples. There was little difference in the moisture content of the peat as well as total C. However, S and N tended to decrease with distance from smelters (Figure 3.2 and Table 3.1). The C:N (Table 3.1) was lower in all impacted sites (mean of 23.84) compared with the reference sites (Cartier Forest and Lawn) which had an average of 46.35. A PCA of chemistry by site (Figure 3.2) highlights the generalized effect of distance and the increase in metals within sites closer to historic smelting

activities with Axis 1 explaining 62.46% of the variation. Variability in samples along axis was driven primarily by unique peat chemistry at Rockcut (RC) relative to the other sites.

3.4.2 Gas Production

Rates of methane and CO_2 production were dependent on distance from smelting activities and depth (Figure 3.3). On average, carbon mineralization as methane (CH_4-C μg C· g^{-1} dry peat · day^{-1}) was significantly ($p<0.001$) slower in the deep samples by a factor of 8.26x while carbon mineralization as CO_2 (CO_2-C μg C· g^{-1} dry peat · day^{-1}) was also significantly slower ($p<0.001$) at depth by 2.37x. In the middle peat profile depth, 50% more CH_4-C than CO_2-C was produced, while at the lower depth, 2.3x more CO_2-C than CH_4-C was produced. In total, the mean CH_4-C produced was 12.0 μg C/g dry peat compared with 14.6 μg C/g dry peat for CO_2-C. CH_4-C and CO_2-C production were correlated with each other ($r = 0.90$, $p<0.001$) and other controls on methane production were evaluated first by other individual correlations, followed by a forward-backward stepwise regression for the more productive middle peat depth. Most individual chemical components were related to distance and were best described by a full PCA of the chemistry (C, N, S, total and extractable elements not containing NAs, plus peat pH) summarized by site and element of interest in Figure 3.2. It should be noted that methane production in the middle depth was weakly correlated with both total and extractable Fe, Mo, Cu (positive) and Ni (negative). A multiple regression model of methane production in the middle depth (Table 3.2) initially included the first three components of the chemistry PCA (Figure 3.2; components not shown), relative abundance of *mcrA*, von Post hunification, CO_2-C, distance, and peat water content. The final model included PCA components one and three as well as relative abundance of *mcrA*, von Post, and CO_2-C (a proxy for general peat biological activity), with distance, PCA

component two, and water content being removed from the model (Table 3.2) and explained a large amount of variance ($R^2 = 0.96$, $p<0.001$).

3.4.3 Sequencing and Community Composition

Community composition of the methanogens as determined by sequencing of the *mcrA* gene indicated that there were influences of both depth and distance from smelting activities. Figure 3.4 shows a CCA of site and depth-level data; there is clear separation of the deep and middle peat methanogen communities along axis 2. An Adonis test of the deep and middle horizon communities indicated that differences by depth were significant ($p=0.003$). Additionally, sites separated clearly with distance from smelters along axis 1; with more polluted sites falling on the left side of the axis and sites further from historic smelting towards the right. Within site differences in depth often caused more variability than among site differences, and there were no significant effects of individual sites for *mcrA* assemblages.

3.4.4 Sequencing and OTU Assignment

A total of 1,559,739 *mcrA* fragments were sequenced from the twenty samples (average of 77,987 per sample), which was reduced to 621,822 reads following the removal of singletons. Of these reads there were 68,820 unique sequences, which grouped together into 34 OTUs (Table 3.3). Phylogenetic resolution was focused on the family level where distinct metabolic differences among methanogens are most prevalent. There were fewer classified sequences in deeper samples and impacted sites tended to also have a considerably larger number of unidentified OTUs (Figure 3.5). At CLM nearly all sequences could be classified (97.3%) while at MGD virtually none (0.03%) could be identified at the family level. In addition to having higher overall OTUs classified, the deeper peat at the two reference sites CL and CF were

dominated by the family Methanoregulaceae (hydrogenotrophic/CO_2-reducing methanogens) and Methaosarcinaceae and Methanosaetaceae (both acetoclastic) became more abundant in the middle depth horizon. In contrast, classifiable taxa (at the family level) in the smelter –impacted sites were dominated by both families (Methaosarcinaceae and Methanosaetaceae) within the order Methanosarcinales, represented in varied proportions. However, the predominant component of the communities of the impacted sites was the unidentified sequences, which also extended to higher taxonomic classifications (e.g. order and class) and thus was not just due to having conducted analysis at the family level. Two of the impacted sites had *Methanomassiliicoccus* present, however, abundance was low (0.26% in DLM and 0.0076% in ASM) and aren't considered a significant component of these communities.

3.4.5 Methanogen Diversity metrics and Indicator Species Identification

Most sites and depths had ca. 2/3 of the total OTUs detected in the global analysis (Chapter 2), and in general there were more OTUs in the middle depth compared to the deep peat (Table 3.4). Additionally, middle samples tended to have higher Shannon diversity and higher evenness than the deep samples. The OTU richness and Shannon diversity tended to decrease with distance from smelters while the communities became less even (Table 3.4). However, for all of these measures, variability among and within sites was high, and there were no statistically significant patterns. Indicator species analysis highlighted that OTUs 9, 16, 17, 18, 20, 27, and 32 (Table 3.3) were responsible for a substantial amount of variability in communities across the middle depth samples, and in all cases except two (OTUs 17 and 32), were present in all middle samples across sites. For the deep peat, OTU 26 was a good predictor of that depth but was only found in half of the deep samples, meaning if it was present it was a deep sample but its absence couldn't

rule out the depth of a sample. There were fewer indicator OTUs that were good predictors of individual sites with OTU 23 and 25 predicting CL well and OTU 29 predicting BL (present in all samples from these sites and were relatively unique to the site).

3.4.6 Phylogenetic Tree and OTU Table

Phylogenetic analysis (Figure 3.6) indicated that OTUs belonging to six orders were present across the Sudbury peatlands. The most abundant organisms were concentrated in the Methanomicrobiales, Methanosarcinales, and Methanocellales orders. A number of OTUs were grouped into orders and families that were not identified in the taxonomy assignment represented in Figure 3.5, namely those OTUs in the Methanobacteriales and Methanococcales, but also a number of the Methanomassiliicoccales; based on low relative abundance values but numerous OTUs clustering to this order phylogenetically. Four OTUs did not cluster to any known order, however sequences retrieved from clones of environmental samples (Table 3.3) show that there are ecologically relevant species in other systems that are closely related to these sequences.

3.5 Discussion

3.5.1 Metal Impacts on Site Chemistry

In line with previous studies (Hutchinson and Whitby, 1974; Freedman and Hutchinson, 1980; Gignac, 1987; SARA Group, 2001), this work shows that the impacts of historic and continued metals and S deposition persist in local peatlands and have altered the methanogen communities, site chemistry, and the potential for methane production. Our first hypothesis was supported in that impacted sites have higher concentrations of S, Ni, and Cu associated with smelting activities and concentrations tended to decrease within the peat profile (i.e. depth). In contrast to

our first hypothesis peat pH increased in impacted sites, likely due to the *Sphagnum* loss in the surface layers as documented by Barrett and Watmough (2015), since previous studies have shown that *Sphagnum spp.* are intolerant of high levels of metals (Gignac, 1987). While the focus of this work was not directly on the plant community. This loss in *Sphagnum spp.* led to fens that tended to be more sedge-dominated, which supports research that has demonstrated that sedges colonize disturbed peatlands (Waddington et al., 1996; Juutinen et al., 2010). Along with the plant community shifts, the peat quality also changed in the impacted sites. The von Post measures of peat tended to increase with level of impact and it appears that these sites have undergone a loss of labile carbon in the past decades; however, it is unclear whether this C loss is due to alleviation of decomposition constraints imposed by *Sphagnum* and/or extreme SO_4 deposition stimulating anaerobic microbial respiration. Current levels of total metals remain comparable to past studies indicating that loss of metals from the system is a slow process and as work by Szkokan-Emilson et al. (2013) indicates is likely tightly tied to rainfall events and water table fluctuations within the peatland profiles.

3.5.2 Methane and carbon dioxide emissions

Greenhouse gas production in the form of methane and CO_2 were both lower in impacted sites, and site chemistry (a proxy for distance) appeared to be a good predictor of potential methane production. Micronutrient availability (Mo, Co, Fe, Ni) has been shown to limit methane production in various systems (Mudhoo and Kumar, 2013; Krishna and Gilbert, 2014; Evranos and Demirel, 2015), but it appears that when provided in excess and together, they may be inhibitory. However, each micronutrient had weak correlations with methane and CO_2 production indicating that under high concentrations in a mixed from it is nearly impossible to

isolate the role each of these elements may be playing in GHG production unless supplemented individually (Hu et al., 2008; Evranos and Demirel, 2015; Mao et al., 2015). Additionally, Cu in these sites is at levels known to be toxic to methanogens, and there is potentially direct competition with sulfate reducing bacteria (due to S addition), both of which likely negated any positive effects of micronutrient availability if there were any (Karri et al., 2006).

3.5.3 Methanogenic Assemblages

The family Methanoregulaceae, which are abundant world-wide (Oren, 2014), were more abundant in reference sites, whereas the impacted sites had higher relative abundance of the metabolically diverse order Methanosarcinales. Finally, in support of our last hypothesis, we found that peat chemistry, microbial community composition, and methane production in deeper peat often varied less with distance than the 30 cm depth did, but overall results still indicated a trend for differences in the impacted sites compared to the reference sites even at a depth of 70-100 cm. The methanogen community composition has been investigated in many contexts, but the impacts of high metals and sulfur deposition are not understood. In a typical peatland system differences in methanogen community structure are subtle (Mackelprang et al., 2011; Godin et al., 2012; He et al., 2015); however, we found a major difference in methanogen communities across our gradient of historic smelting activities. The Cartier (reference) sites had communities that were more similar across depths and in line with other peatland methanogen communities, with most of the OTUs identifiable at the order level (Steinberg and Regan, 2008; Bridgham et al., 2013). These methanogens were predominantly hydrogenotrophic and belonged to the Methanoregulaceae family. While we found fewer known families in the deep samples from these sites, this is not uncommon in other undisturbed peatland methanogen communities

(Galand et al., 2002) and differences are often considered at the order level. In contrast to this, the impacted sites we sampled had a completely different community profile at both depths.

3.5.4 Links between emissions and community assemblages

The abundance of *mcrA* (measured via qPCR) was loosely correlated with methane production. Transcript work tends to identify microbial processes more accurately (Hierro et al., 2006), but our results still indicate that overall methanogen abundance is a good predictor of methane production, meaning if methanogens are present they are likely active. The methanogen community composition varied across distance and with depth, however there is little evidence that these differences played a role in lower methane production in impacted sites. This is supported by the high methane production rates in the impacted sites AS and MG, which had dramatically different community composition than the reference CF/CL sites that also produced high levels of methane and CO_2. The best predictor of methane production across sites was CO_2-C and indicates that overall microbial activity and substrate quality (dead organic matter (OM) and active plant inputs) plays an important role in supporting rapid methane production. This idea is reinforced by other work that has found that both labile C and overall microbial activity predicts methane emissions well (Joabsson and Christensen, 2001; Turetsky et al., 2014). Effectively methanogens need substrates to grow on and in most cases are dependent on bacterial byproducts in synergistic syntrophic relationships (Hutchin et al., 1995; Juottonen et al., 2005; Sieber et al., 2010).

3.5.5 Novel OTUs

In contrast to most other peatland studies it appears that the Sudbury sites, under high pollution impact and over a long time, have developed unique communities through a bottleneck effect;

evidenced by impacted sites having substantial losses of known hydrogenotrophic methanogens and favoring methanogens with more diverse metabolic pathways. This fits with ecological principles that would predict survival of more generalist/adaptive species under intense stressors (van Tienderen, 1997; Clavel et al., 2011). Even more importantly, in the impacted sites the majority of OTUs were unidentified to the order level indicating that a large proportion of the methanogens in these sites are either very uncommon or potentially difficult to culture. Many of the unknown OTUs are from clones of *mcrA* sequences taken from primarily aquatic and wetland environments (Table 3.3), indicating that these unknown methanogens likely play an important role in other systems. Taken together this indicates that the methanogen reference library is missing a large number of potential methanogens as highlighted in other work (Juottonen et al., 2006; Sakai et al., 2007). While we found that these unknown methanogens were less active in our sites, there is potential that they may play an important role in other ecosystems where stressors of high metal concentrations are not an issue.

3.6 Conclusions

Our work demonstrates the impact of mining practices and associated air pollution on peatland chemistry and vegetation and in turn the implications these play in potential methane production and methanogen community structure. Deposition patterns remain consistent with past work and the lasting effects can be seen, even as environmental pollution has been mitigated. Methanogen community structure has been dramatically modified leading to largely unknown community composition and potentially novel species in these sites that can lead to the discovery of species relevant to other impacted sites near other smelters in Canada, Russia, Scandinavia, the US and globally (see Kozlov and Zvereva 2007). The importance of mitigating high levels of metals

deposition into peatlands cannot be understated, and while the potential for GHG emissions is reduced the other ramifications, such as loss of C stores, far outweigh this potential gain. This work also indicates that plant community structure plays an important role in methane emissions and methanogen communities, although it was difficult to parse out specific linkages between the two, or to separate these effects from those caused by heavy metal input and altered peat chemistry. More research is required to identify thresholds of metal loadings in peatlands using individual elements and combinations to more systematically approach the questions surrounding the role these play in natural systems. It is also evident that more work needs to be done to identify unknown methanogens present in these impacted sites, either through more intensive culturing efforts or by using modern molecular techniques.

3.7 Tables and Figures

Table 3.1 General site characteristics. Mean values (n = 6) and dominant vegetation for sites sampled around Sudbury. Significant differences are not shown due to a strong interaction effect of site and depth. *Distance is calculated from the centroid coordinates of the three smelters: Copper Cliff, Falconbridge, and Coniston.

Site	Site Name	Distance*	Dominant Vegetation	% Moisture	Von Post	Peat pH	%C	%N	%S	C:N	Ni (mg/kg)	Cu (mg/kg)
DL	Daisy Lake	6.80	*Chamaedaphne calyculata* (leatherleaf)	86	6.17	5.1	40.4	2.05	0.69	19.72	2401	737
LU	Laurentian	8.17	*Chamaedaphne calyculata* (leatherleaf)	89	7.67	4.7	48.4	1.79	0.32	27.05	410	189
BL	Broder Lake	13.58	*Juncus canadensis* (Canadian rush)	88	7.67	4.9	39.9	1.96	0.69	20.33	478	509
CW	Clearwater	20.16	*Chamaedaphne calyculata* (leatherleaf)	88	7.5	3.7	50.1	1.56	0.32	32.12	183	316
LL	Long Lake	20.46	*Chamaedaphne calyculata* (leatherleaf)	78	7.67	5.1	38.4	1.78	0.68	21.54	694	590
RC	Rockcut	24.04	*Myrica gale* (sweet gale)	73	7.67	4.2	25.8	1.34	0.45	19.22	140	99
AS	Ashigami	26.45	*Carex magellanica* (boreal bog sedge)	87	7.33	4.6	42.5	2.02	0.65	21.06	217	245
MG	Matagamasi	30.98	*Chamaedaphne calyculata* (leatherleaf)	88	7.17	4.2	44.5	1.50	0.33	29.67	127	160
CL	Cartier Lawn	50.12	*Sphagnum rubellum* (red bog moss)	94	5.00	3.5	44.7	0.89	0.15	50.19	19	21
CF	Cartier Forest	50.21	*Sphagnum fuscum* (rusty peat moss)	90	6.67	3.5	46.8	1.1	0.15	42.5	30	25

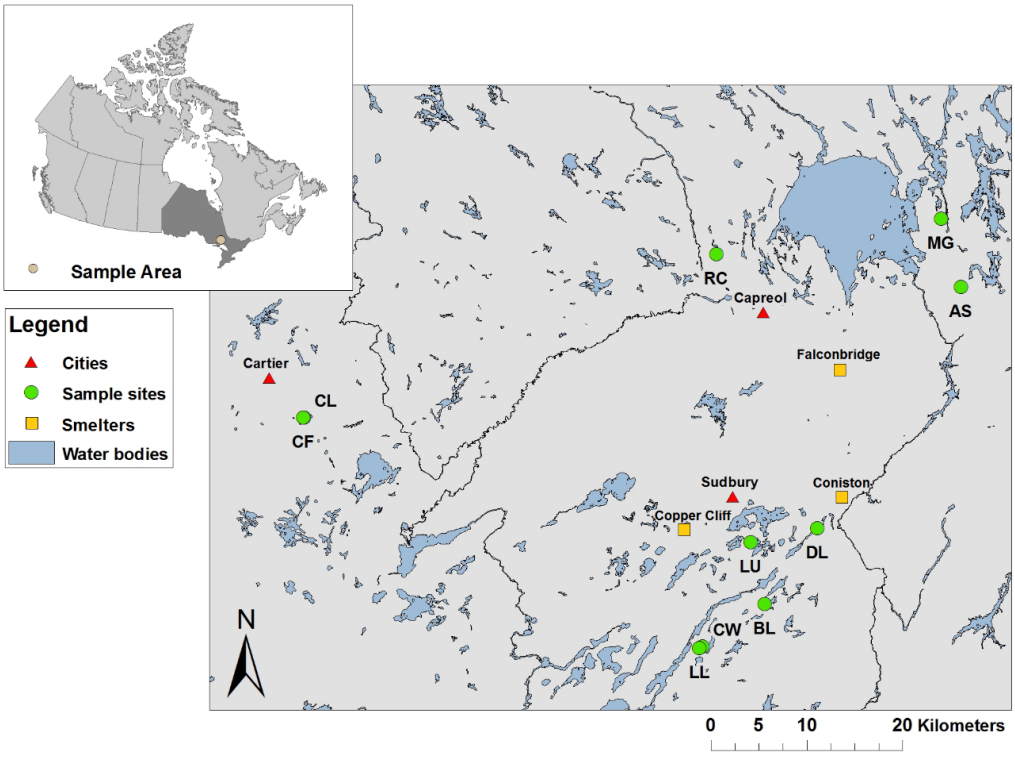

Figure 3.1 Site map. Locations of ten fens selected for sampling relative to the three historic and current smelters in Sudbury, Ontario. The Cartier forested (CF) and Cartier lawn (CL) peatlands were used as reference sites, while other sites were located in watersheds draining into Long Lake (LL), Clearwater Lake (CW), Broder Lake (BL), Lake Laurentian (LU), Daisy Lake (DL), Ashigami Lake (AS), Matagamasi Lake (MG), and Rockcut Lake (RC).

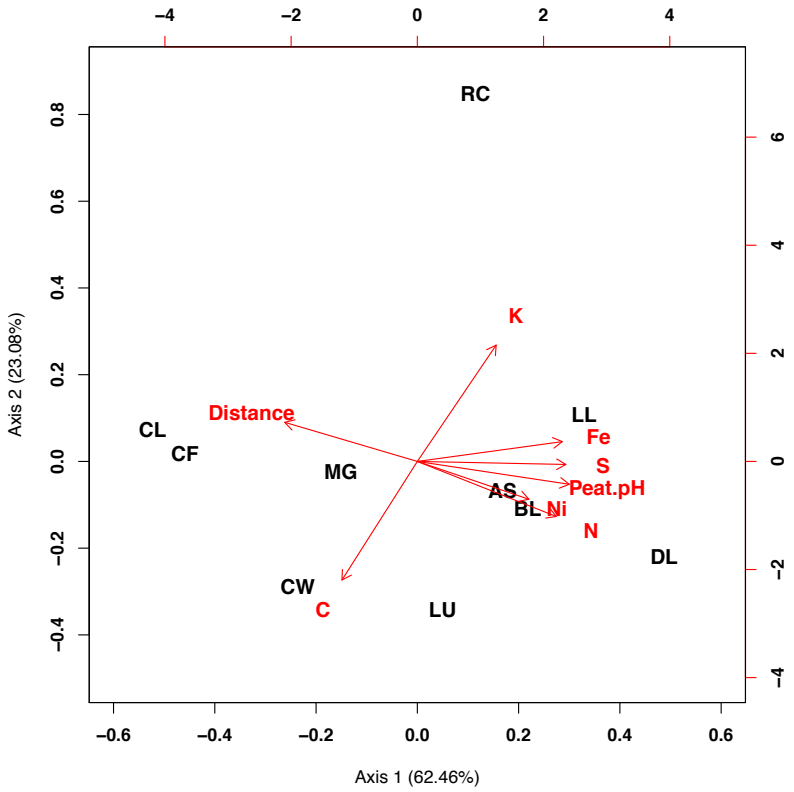

Figure 3.2 Total element chemistry PCA. Representative ordination of total elements by site selected from a full PCA containing all elements. Axis 1 is driven primarily by a pattern of decreased metals with distance from smelting. Distances (km) for sites are as follows: DL (6.8), LU (8.2), BL (13.6), CW (20.2), LL (20.5), BR (24.0), AS (26.5), MG (31.0), CL (50.1), CF(50.2).

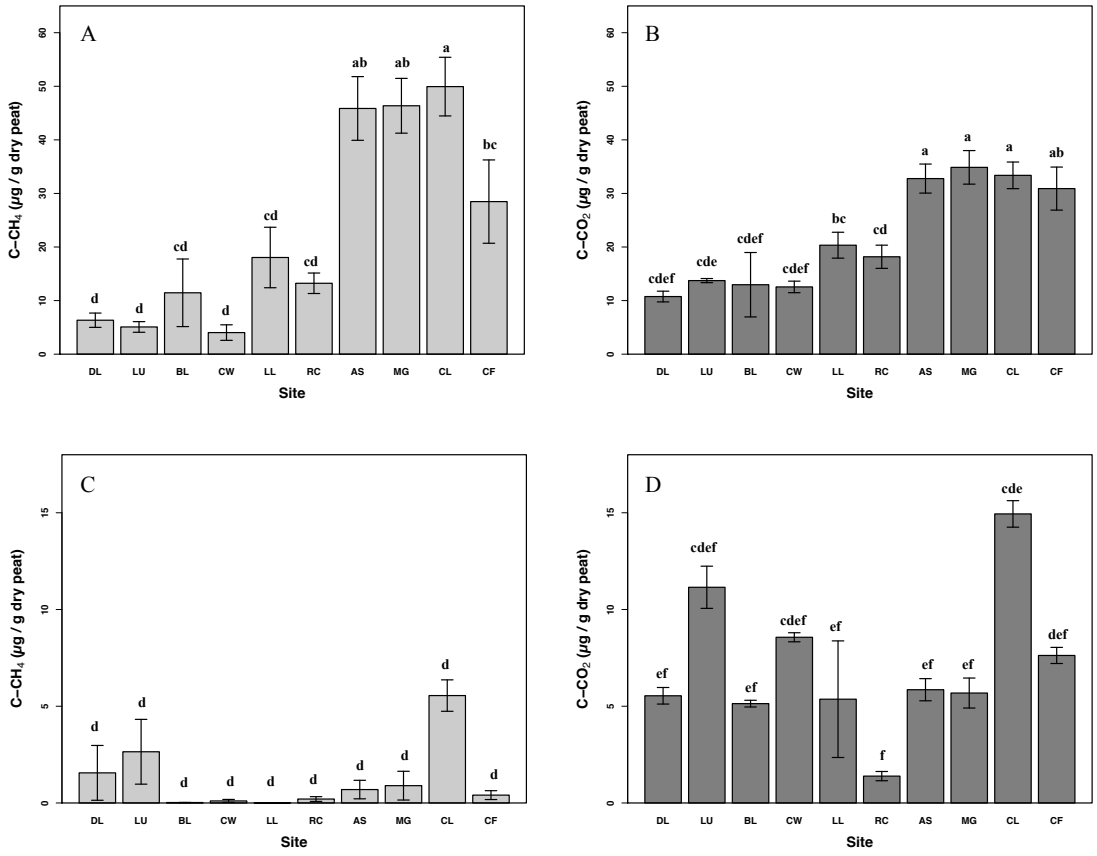

Figure 3.3 Potential greenhouse gas production. Mean production (+/- se) of methane for middle (A) and deep (C) and CO_2 for middle (B) and deep (D) peat incubations conducted over 45 days. Sites are arranged left to right by increasing distance from centroid smelter coordinates. Letters designate significant differences ($p<0.05$) identified in a Tukey's post hoc tests for the interaction of site and depth within both methane (panels A and C) and CO_2 (panels B and D). Distances (km) for sites are as follows: DL (6.8), LU (8.2), BL (13.6), CW (20.2), LL (20.5), BR (24.0), AS (26.5), MG (31.0), CL (50.1), CF(50.2).

Table 3.2 Stepwise regression comparing methane production to principle components of chemistry, *mcrA* qPCR data, CO_2 production, distance and water content. AIC model selection criteria were used and a forward-backward progression for individual steps. The overall model contained 29 complete observations.

Input model:

Methane ~ PCA 1 + PCA 2 + PCA 3 + *mcrA* + Von Post + CO_2-C + Distance + Water Content

Resolved model:

Methane ~ PCA 1 + PCA 2 + *mcrA* + Von Post + CO_2-C

	Estimate	p-value	
Intercept	-31.36	0.000	***
PCA 1	-1.35	0.042	*
PCA 2	2.18	0.029	*
mcrA	0.16	0.000	***
Von Post	2.26	0.050	.
CO_2-C	1.25	0.000	***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Multiple R-squared 0.96
Adjusted R-squared 0.95
F-statistic: 118.7 on 5 and 24 DF, p-value: < 4.095e-16

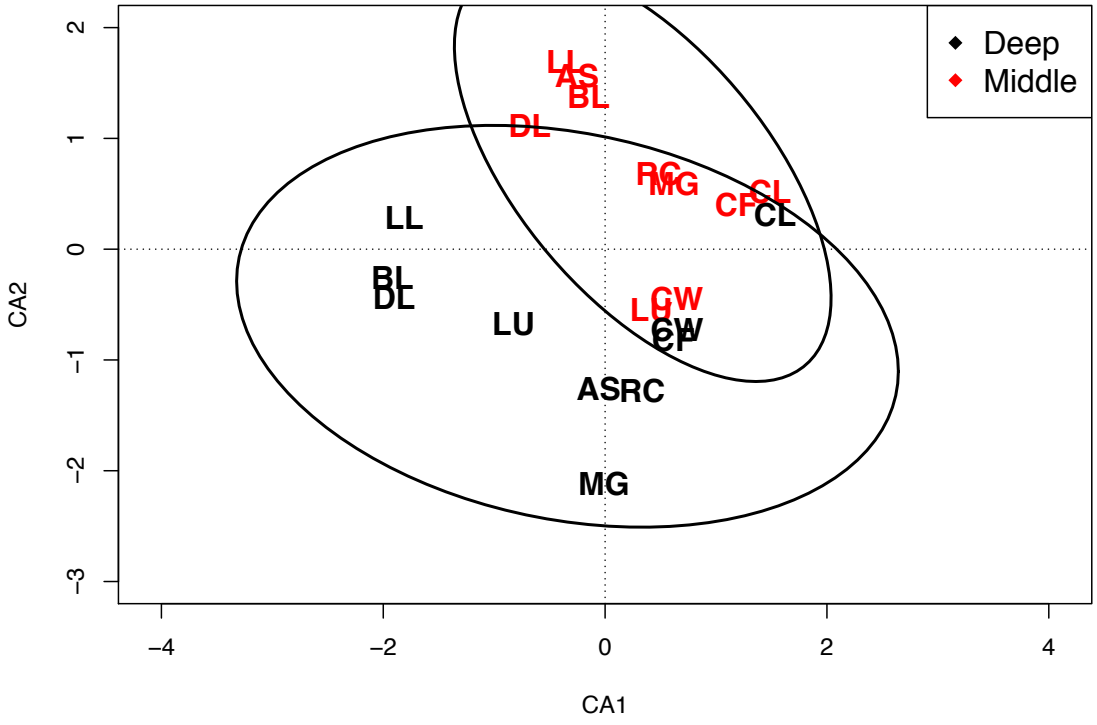

Figure 3.4 CCA of sites by depth for methanogen communities based on *mcrA* metagenomic sequencing. Ordination elippses represnet 95% CI. Distances (km) for sites are as follows: DL (6.8), LU (8.2), BL (13.6), CW (20.2), LL (20.5), BR (24.0), AS (26.5), MG (31.0), CL (50.1), CF(50.2).

Table 3.3 OTU table for the 34 *mcrA* clusters identified in Sudbury peatlands. Numbers represent read counts of individual OTUs by sample and were filtered to remove singletons during data processing. The nearest published match is listed with its accession number, habitat type it was obtained from and the publication. Row totals represent total reads per OTU while column totals give total reads per sample. Distances (km) for sites are as follows: DL (6.8), LU (8.2), BL (13.6), CW (20.2), LL (20.5), BR (24.0), AS (26.5), MG (31.0), CL (50.1), CF(50.2).

Site	DL		LU		BL		CW		LL		RC		AS		MG		CL		CF					
OTU	Middle	Deep	Middle	Deep	Middle	Deep	Middle	Deep	Middle	Deep	Middle	Deep	Middle	Deep	Middle	Deep	Middle	Deep	Middle	Deep	OTU Reads	Nearest Published Match (Accession #)	Habitat	Publication
1	57	18	54	49	55	5	103	15	84	27	117	18	116	38	197	5	49820	10318	19594	2535	83225	*Methanoregula boonei* 6A8 (DQ205189)	Peatland	Brauer et al. 2006
2	59	160	13154	1590	48	83	19191	4412	19	31	59	3420	94	9759	98	10	40	32	887	30368	83514	clone 0912F25 (FR725700)	Peatland	Hunger et al. 2011
3	9	322	203	673	5	52	8	4	114	41	20	1756	19	14461	48	29718	5	6	13	3	47480	clone RFm_Koral_08 (AM746713)	Rice roots	Conrad et al. 2008
4	25	97	12	406	14	229	6	5	14	21859	18	0	16	12	25	1	27	11	18	9	22804	clone OA_B01 (HE774271)	Wetland	Franchini and Zeyer 2012
5	6	7	24	6	12	1	18	649	6	8	2	2	14	73	15	4	18	13884	61	401	15211	clone OA_C08 (HE774276)	Wetland	Franchini and Zeyer 2012
6	10	4	1944	191	17	2	6806	155	17	11	333	11	21	12	1409	1	271	71	8406	323	20015	clone valumaN2-2 (FN565451)	Peatland	Juottonen et al. 2012
7	2194	64	35	39	1099	5	11	9	16570	43	69	4	7889	1530	114	4	64	30	44	25	29842	clone novmcr36 (AF525521)	Lake water	Earl et al. 2003
8	102	8179	1	151	1	768	0	1	1	58	1	1	2	105	0	0	1	0	3	7	9382	clone U3-F08 (KR075367)	Florida Everglades	Bae et al. 2015
9	31	3	1334	90	825	0	4806	261	456	10	3017	9	501	16	3283	0	384	63	5347	367	20803	clone OA1_G02 (KP071406)	Peatland	Cheema et al. 2015
10	260	784	20	8992	12	179	3	0	8	3096	3	7	62	4989	15	0	8	5	3	0	18446	clone OA_G08 (HE774292)	Wetland	Franchini and Zeyer 2012
11	1143	802	10	316	231	478	6	5	87	14625	7	2	16	19	16	2	12	14	16	13	17820	clone: SpM10 (AB570064)	Peatland	Narihiro et al. 2011
12	47	1347	1570	8477	6	1	0	3	50	3	7	2	21	680	9	0	11	3	3	6	12246	clone 185 (GU085013)	Bog lake	Milferstedt et al. 2010
13	3	2	6	5	4	6	7824	1	2	4	3	1	6	5	4	1	158	89	584	1260	9968	clone mcrA34 (JN030390)	Rice paddy	Ma et al. 2012
14	90	2	402	79	229	6	0	2	437	3	920	54	1381	28	519	1	1	1	1	0	4156	clone contig 00131 (KC184915)	Lake water	Denonfoux et al. 2013
15	17	3111	6	5	3	1344	1	1	3	136	0	3	13	8	3	1	3	2	0	0	4660	clone 4-21 (KM273365)	Rice roots	Pump et al. 2015
16	69	12	501	109	162	0	1272	22	80	4	435	58	243	18	2741	0	2944	437	722	79	9908	clone 5-11 (KM273372)	Rice roots	Pump et al. 2015
17	136	3	1	0	1137	0	0	1	529	0	2	0	477	61	6	0	0	5	2	0	2360	*Methanobacterium lacus* strain AL-21 (CP002551)	Peatland	Cadillo-Quiroz et al. 2014
18	32	1	1	0	383	9	1	1	1486	2	1	0	212	70	2	0	2	2	1	1	2207	clone 329_19g (KT225456)	Wetlands	Söllinger et al. 2016
19	199	16	1876	269	18	1	18	2	172	3	1014	18	153	74	148	1	0	1	40	1	4024	clone 0913F77 (FR725498)	Peatland	Hunger et al. 2011
20	405	22	40	33	477	0	2	2	1834	0	23	45	1871	107	34	1	12	1	8	2	4919	clone 2312F30 (FR725816)	Peatland	Hunger et al. 2011
21	0	688	0	0	1	39	0	1	0	1	0	0	0	0	1	0	0	0	1	1	733	clone G10IITuc78 (FJ715523)	Lake sediment	Santana et al. 2012
22	18	1	0	0	0	0	0	1	9	0	0	0	6	461	0	0	1	1	0	0	498	clone: SpM14 (AB570068)	Peatland	Narihiro et al. 2011
23	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	456	142	0	0	600	clone Lak25-ML (AJ853831)	Peatland	Juottonen et al. 2005
24	2	1	0	3	0	0	0	0	255	0	0	0	0	64	3	1	1	0	1	0	331	clone mcrA105 (JQ618217)	Rice rhizosphere	Xu et al. 2012
25	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	405	74	0	0	480	clone MB03-27 (EF165655)	Peatland	Metje and Frenzel 2006 Unpublished-Direct Submission
26	0	9	0	53	0	24	0	0	1	320	0	0	1	0	0	0	0	0	0	1	409	No Match Published closest ident 87% clone M2_40 (JX942683)	Estuary	Li et al. 2012 Unpublished-Direct Submission
27	13	0	5	0	92	0	3	1	35	0	2	0	42	7	9	0	3	0	1	0	213	clone ML-ACH19 (KJ464155)	Freshwater marsh	Lin et al. 2015
28	5	1	0	0	0	0	0	1	0	0	0	0	0	153	0	0	0	0	0	0	160	clone M_mcrA-11 (JQ406852)	Petroleum water	Li et al. 2012
29	1	0	0	0	1	34	0	0	0	0	0	0	0	0	0	0	0	0	0	0	36	clone mcrA137 (JQ618233)	Rice rhizosphere	Xu et al. 2012
30	0	0	0	0	12	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	15	clone 174 (GU085002)	Bog lake	Milferstedt et al. 2010
31	13	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	14	*Methanomassiliicoccus luminyensis* strain B10 (HQ896500)	Human feces	Dridi et al. 2012
32	0	0	9	0	0	0	0	0	3	0	6	0	5	0	4	0	0	0	0	0	27	No Match Published closest ident 84% clone M2_11 (JX942659)	Estuary	Li et al. 2012 Unpublished-Direct Submission
33	0	0	0	0	0	0	19	0	0	0	0	0	0	0	0	0	0	0	0	0	19	clone 42-GD3.8 (KF228277)	Rice paddy	Bao et al. 2014
34	5	0	0	0	1	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	11	clone 6 (KJ609576)	Soil	Holmes et al. 2014
Sample Reads	4951	15656	21208	21537	4845	3266	40098	5555	22272	40285	6061	5411	13190	32750	8703	29751	54647	25192	35756	35402				

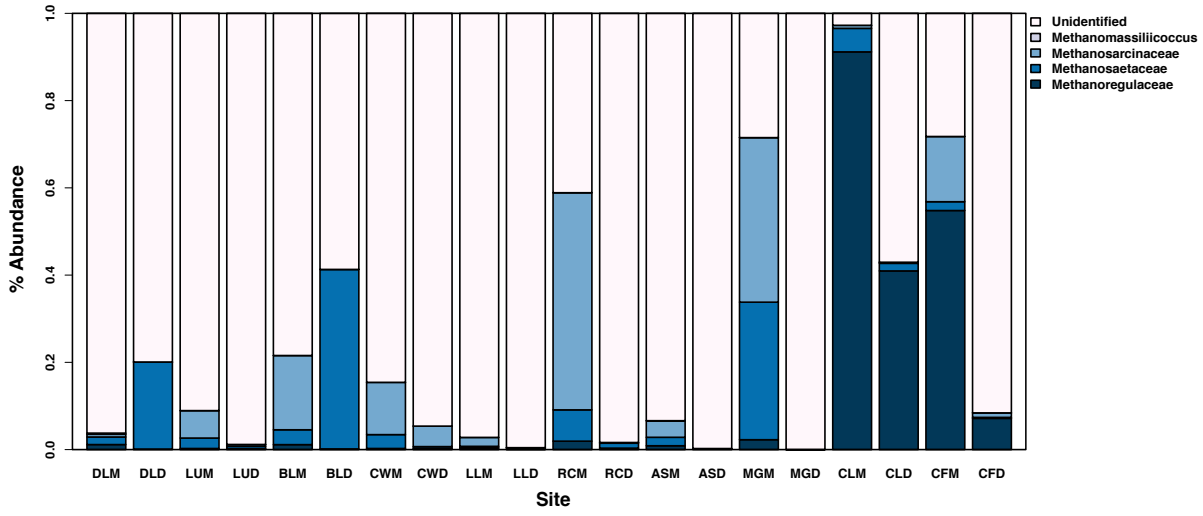

Figure 3.5 Family level *mcrA* abundance. Relative abundance of *mcrA* in ten sites and two depths (M-"middle, D-"deep"). Sites are arranged in increaseing distance from smelting (L-R). Values are the average of three DNA extractions at each site/depth combination homogenized for sequencing. Note Methanomassiliicoccus is only 0.26% in DLM, and 0.0076% in ASM, all other sites had 0% abundance. Distances (km) for sites are as follows: DL (6.8), LU (8.2), BL (13.6), CW (20.2), LL (20.5), BR (24.0), AS (26.5), MG (31.0), CL (50.1), CF(50.2).

Table 3.4 Methanogen community summary. OTUs represents the number of total OTUs present in each depth and site out of a total of 34 *mcrA* OTUs identified. Shannon diversity and evenness were calculated from the OTU abundance table. Relative abundance of the *mcrA* was determined using qPCR and is calculated as the relative abundance per gram of dry peat normalized to extraction yield. Distances (km) for sites are as follows: DL (6.8), LU (8.2), BL (13.6), CW (20.2), LL (20.5), BR (24.0), AS (26.5), MG (31.0), CL (50.1), CF(50.2).

	25cm Deep				75cm Deep			
Site	**OTUs**	**Shannon Diversity**	**Evenness**	**RA *mcrA***	**OTUs**	**Shannon Diversity**	**Evenness**	**RA *mcrA***
DL	27	1.9	0.6	85.5	25	1.5	0.5	4.95
LU	22	1.4	0.4	22.0	21	1.4	0.5	6.52
BL	25	2.1	0.7	54.0	19	1.7	0.6	0.61
CW	18	1.4	0.5	9.8	23	0.8	0.2	0.22
LL	25	1.1	0.3	64.9	20	1.0	0.3	0.01
RC	23	1.6	0.5	23.3	17	0.9	0.3	0.59
AS	27	1.5	0.4	104.6	24	1.5	0.5	9.81
MG	23	1.6	0.5	82.5	14	0.0	0.0	0.00
CL	23	0.4	0.1	65.0	22	0.9	0.3	5.20
CF	22	1.2	0.4	25.5	18	0.6	0.2	7.28

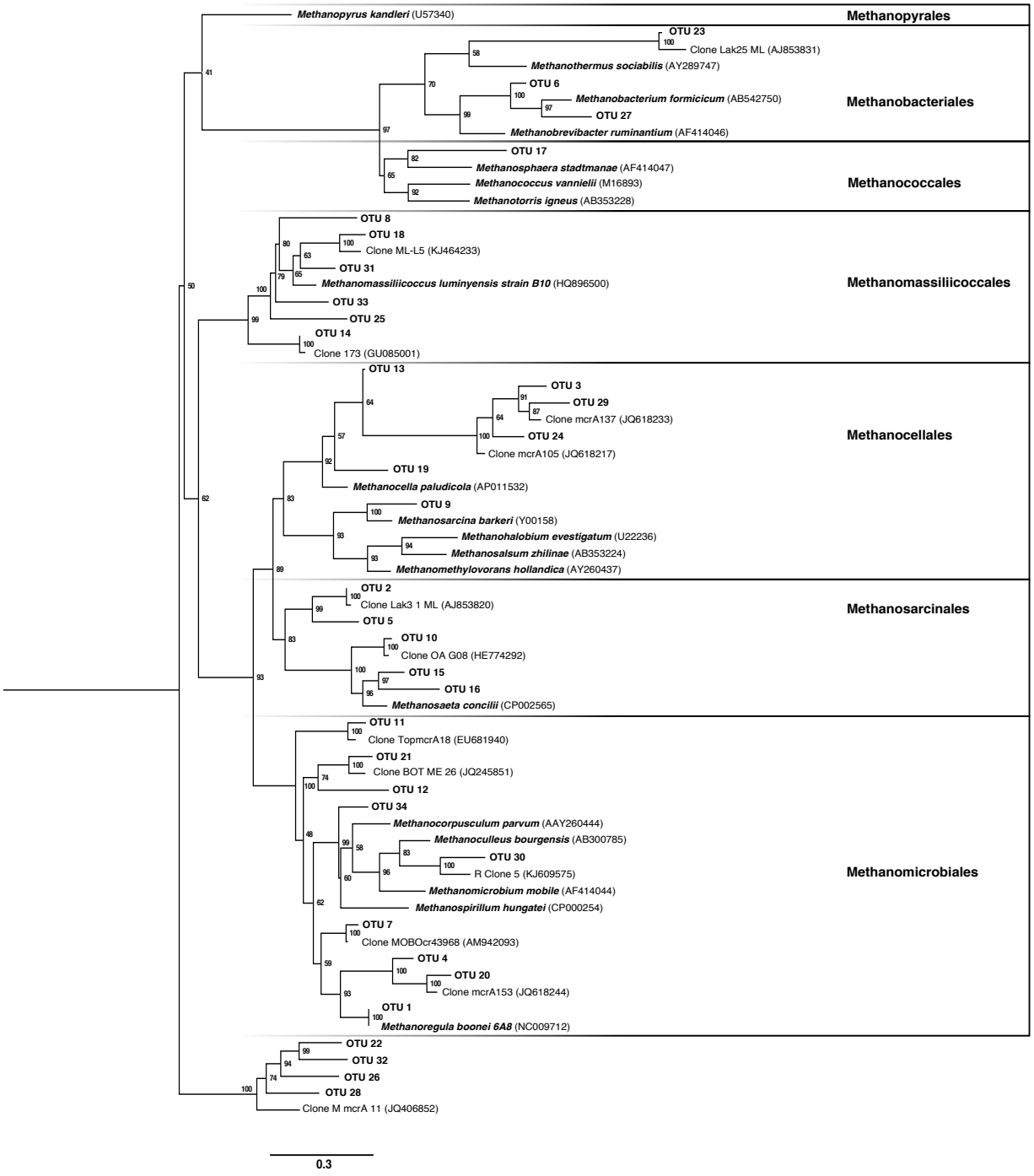

Figure 3.6 Phylogenetic tree of *mcrA* gene. Maximum likelihood tree constructed from ten sites surrounding Sudbury, ON. The tree was constructed using a Bayesian model with 1000 replicates and bootstrap values are shown on each node. Branches were grouped into methanogen orders according to cultured sequences and the tree was rooted to an ANME I clone (AY327048) *mcrA* sequence outgoup to ensure all OTUs were methanogens.

Chapter 4 Environmental enrichments for novel methanogens: Approaches for obtaining uncultured organisms in the age of rapid sequencing

4.1 Abstract

Methanogens are among the oldest forms of life on Earth and are detectable in a wide range of environments, but our knowledge of their overall diversity and functioning is limited. Peatlands in particular host a broad range of methanogens that contribute large amounts of methane to the atmosphere on an annual basis, but are largely underrepresented in pure cultures. Here we anaerobically enriched peat with common growth substrates, supplements, and antibiotics with the intent of identifying novel methanogen sequences and potential growth conditions. Over the course of three years we were able to obtain 28 new *mcrA* sequences from taxa that have remained previously uncultured and undescribed beyond distantly related clones or sequences detected in environmental samples. Evidence suggests that novel methanogens, representing five of the seven known orders, were capable of growing on H_2 as well as acetate, and at temperatures ranging from 6 °C to ca. 22 °C. Methods involving the use of ampicillin proved useful and obtaining high methane production in the absence of H_2 was difficult. Our results also indicate that many methanogens may rely on bacterial symbionts (commonly *Clostridium spp.*) and enrichments will be a useful intermediary between maker-gene detection and isolation, allowing us to broaden our understanding of methanogen physiological ecology and bolstering our reference sequence library to support the plethora of *mcrA* and rRNA gene based community fingerprinting studies. Due to the relative difficulty in isolating anaerobic microorganisms, and

methanogens in particular, we conclude that enrichments and modern sequencing methods are a way forward in developing an understanding of methanogen diversity and functioning in a variety of ecosystems.

4.2 Introduction

Methanogens are thought to be some of the first life to have evolved on Earth and are found globally in environments ranging from the stomachs of ruminants, termite hindguts (Schlesinger and Bernhardt, 2013), and human intestinal tracts (Whitford et al., 2001; Dridi et al., 2012) to anaerobic digesters (Mudhoo and Kumar, 2013), ocean sediments (Newberry et al., 2004) and wetland soils (Basiliko et al., 2003; Juottonen et al., 2005; Godin et al., 2012). As their name implies, methanogens produce methane, a potent greenhouse gas, giving them particular interest for research on global climate change. While methanogens play a significant role in greenhouse gas production, both naturally (peatlands, etc.) and from anthropogenic sources (rice cultivation, cattle, etc.), there are relatively few pure culture representatives for such a diverse phylogenetic group (Liu, 2010). This presents a challenge when trying to use modern DNA-fingerprinting based approaches to understand both the diversity as well as the functioning as methodologies rely on a library of known sequences to compare unknowns against. This lack of cultured representatives is in large part due to the relative difficulty in isolating and maintaining methanogens in comparison to many bacteria, driven by the fact that methanogens are anaerobic organisms (Narihiro and Kamagata, 2013).

Anaerobic culturing is rooted in work by Hungate (1969) who first developed methodologies of excluding oxygen and growth on reduced medias. These methods were further developed by Balch and Wolfe (Balch and Wolfe, 1976; Balch et al., 1979) and are summarized

further by Sowers and Noll (1995). In the past two decades, culturing in general, and especially anaerobic culturing, has been much overlooked with the rapid development in sequencing technologies beyond that of the Sanger sequencing method. This rapid development was prompted in large part by the human genome project around 2001 (Collins et al., 2004). Since then, cost has decreased substantially and modern high-throughput sequencing has grown exponentially making discoveries using mass datasets possible (Earl et al., 2003; Steinberg and Regan, 2008; Borrel et al., 2011; Sinclair et al., 2015). For example, recent metagenomic work has indicated that there may be microorganisms outside of the Euryarchaeota capable of methanogenesis (Evans et al., 2015); yet, all currently cultured methanogens are in the Euryarchaeota. This apparent contradiction between culture-based and molecular-based work highlights the fact that there is an evident need for enrichments and culture data that will complement and support the sequencing data.

Even in the age of next generation sequencing, some researchers continue to focus on culturing based methods to answer questions that cannot simply be answered by mass DNA sequence information and data processing alone (e.g. Cadillo-Quiroz et al. 2008; Dedysh 2011; Harbison et al. 2016). Anaerobic culturing is a difficult and lengthy process requiring specialized lab equipment, but the use of enrichment techniques in combination with sequencing allows us to determine broad constraints on growth for a particular habitat while obtaining important genetic information with relative ease. In this study, we attempted to enrich and isolate novel methanogens while at the same time leveraged molecular tools to gain progressively more information at each step of the process. The overall goals of this work were to: (1) identify local peatlands (Sudbury, ON) with potentially novel organisms with global relevance, (2) use a series

of enrichments under varied conditions intended to promote methane production and growth of methanogens, (3) track bacterial and methanogen communities in enrichments via molecular fingerprinting approaches to determine the complexity and composition in successful enrichments, and (4) attempt to isolate the methanogen species in pure culture on an artificial growth media.

4.3 Methods

4.3.1 Site Selection and Sampling

Four study sites in Northern Ontario were sampled in the summer of 2014. Past molecular work by Godin et al. (2012) that indicated the presence of novel methanogens (high abundance of members of the Rice Cluster II clade) in the White River, ON (48°21'15.19" N, 85°20'14.10" W) peatland lead us to conduct initial enrichments with this peat. Sites in and around Sudbury, ON were screened in more depth using TRFLP (below, 4.3.3) profiles and two additional sites were selected for this study including "Cartier Forest" (46°39'46.72" N, 81°31'15.00" W), Clearwater" (46°22'9.40" N, 81°3'43.9" W), and "Daisy Lake" (46°27'14.57" N, 80°52'34.99" W). In each case, peat was collected anaerobically by digging a hole to approximately 30 cm deep, or just below the water table. A 125 ml glass Mason jar was then submerged and peat was packed into the jar and capped. Peat was stored on ice during transport to the lab and was either refrigerated at 6 ºC or frozen at -20 ºC prior to starting enrichments. Figure 4.1 depicts the general workflow post sampling that is further elaborated on below.

4.3.2 DNA Extraction and PCR Amplifications

Subsamples of peat from each site were extracted with the Power Soil™ DNA kit (MoBio, USA), following the manufacturer's protocol modified to include 0.5 g of peat in the bead tube and three washes with 500 μl of 5.5M guanidine thiocyanate (humic removal) prior to the addition of the C5 solution. Enrichment DNA (below) extractions were also done using the Power Soil™ DNA kit and used 1 ml of enrichment media in place of peat. Extractions followed the manufacturer's protocol with the addition of a 10 minute heat treatment at 65 °C prior to bead beating. PCR amplifications were done using multiple primer sets. The 16S rRNA 1AF(5'-TCY GKT TGA TCC YGS CRG AG-3') – 1100R (5'-TGG GTC TCG CTC GTTG-3') pair (Hales et al., 1996) was used to broadly screen for methanogens and for TRFLP analysis. A methanogen specific *mcrA* gene was amplified using the mlasF-mod (5'- GGY GGT GTM GGD TTC ACM CAR TA-3') – mcrAR (5'-CGT TCA TBG CGT AGT TVG GRT AGT-3') (Luton et al., 2002; Juottonen et al., 2006; Angel et al., 2012). Bacterial PCRs were conducted using the broad spectrum 16S 27F-1492R primer pairing (Suzuki and Giovannoni, 1996). PCRs were done using ThermoFisher Phire Green Hot Start II master mix following the manufacturer's recommendations for amplification times with annealing temperatures of 55 °C, 56 °C, and 61°C for the 1AF-1100R, mlasF-mod- mcrAR, and 27F 1492 R, respectively. PCR products were confirmed by electrophoresis in a 1% agarose gel prior to downstream analyses.

4.3.3 Terminal Restriction Fragment Length Polymorphism (TRFLP)

A PCR was done using the 1AF-1100R primer pairing described above with a 6-FAM fluorescent tag added to the terminal (3') end of the reverse primer. Following successful amplification, PCR product was cleaned with a GenElute™ PCR clean up kit (Sigma Aldrich)

following the manufacturers protocols. Cleaned fluorescently labeled DNA fragments were digested with HhaI and Sau96I enzymes (New England BioLabs) at 37 °C for 120 min and then 65 °C for 20 min. Fragments were sent to the University of Guelph Laboratory Services where samples were purified and analyzed on an Applied Biosystems 3730 DNA Analyzer. Peak locations were adjusted by 4 base pairs (bp) to correct for systematic read error, quality filtered to peaks >1% abundance and between 50 and 800 bp, and matched to a set of user defined known peaks based on prior TRFLP/cloning studies (Vianna et al., 2009; Cadillo-Quiroz et al., 2010; Yrjälä et al., 2011; Angel et al., 2012) using a custom R script that binned peaks into user defined ranges.

4.3.4 Enrichment Technique

Methanogen enrichments were set up following the methods of (Bräuer, Cadillo-Quiroz, et al., 2006; Bräuer, Yashiro, et al., 2006). Subsamples of peat from selected sites (above) were placed into a Coy anaerobic glove box (Grass Lake, MI) with water Balch style tubes (Chemglass CLS-4209-10), and butyl rubber stoppers and crimp rings. Peat and sterilized water were added to tubes in a 1:9 ratio, respectively. Tubes were then capped and crimped to seal before being removed from the glove box. Peat and water slurries were attached to a vacuum manifold and bubbled horizontally with an 80/20 mixture of N_2/CO_2 gas for five minutes to ensure anaerobic conditions. Amendments and growth substrates (SI Table 5) were then added to the tubes using anaerobic culturing techniques. Briefly, tube caps and amendment bottles were flamed to sterilize, then a new sterile 1 ml syringe and 25-gauge needle were opened and attached. A metal cannula fitted with a 0.45-micron inline filter was heat sterilized and the syringe needle was placed inside the cannula and flushed with N_2 by "pumping" the syringe 5 times. A volume of N_2

was added to the amendment bottle and the necessary amendment volume, typically 0.1 ml per enrichment tube, was extracted and transferred to the appropriate enrichment tubes. This process was repeated to obtain the desired mix of amendments in a particular enrichment tube (e.g. Tables 1-3). For tubes that required H_2 in the headspace, an inline 0.45-micron filter was attached to flexible rubber tubing and a needle was fitted to the end. Hydrogen gas was then added at ten PSI to re-flamed tubes for a five second count. Enrichments were then kept in the dark at either room temperature (ca. 22 °C) or refrigerated at 6 °C for varied durations. Methane was measured periodically (below) to determine when methanogens had become abundant. This process was repeated numerous times to determine the optimal conditions and mixtures of amendments. For more detailed methodological explanations of anaerobic enrichment processes see Wolfe (2011).

4.3.5 Methane Measurements

Methane production was measured periodically to determine if enrichments and/or cultures were active. In each case, a sterile 1 ml syringe fitted with a 27 gauge needle was used to sample 0.5 ml of headspace gas from the Balch tubes. Prior to headspace sampling the syringe was flushed with N_2 gas passed through a 0.45-micron filter ending in a heat sterilized stainless steel cannula. Samples were injected directly into the sample port of a SRI 8610C gas chromatograph (Torrance, CA) fitted with a flame ionization detector. Both methane and CO_2 (after reduction in an in-line methanizer) were detected as methane and instrument parameters included a column temperature of 105 °C and a run time of three minutes.

4.3.6 Transfers to Media

In an attempt to isolate methanogens in pure culture, or at least maintain stable mixed culture lines, enrichment substrate was anaerobically transferred to prepared growth media from Bräuer (2006a; 2006b) and references cited therein (SI Table 5). Media containing minor and major metals was prepared anaerobically from stock solutions, bubbled under N_2 for 20 minutes and added to the glovebox. All materials were placed in the glovebox 24 hr beforehand. Sterilized Balch tubes first had 5 or 10 ml of media transferred to tubes, which were then capped with 20 mm thick butyl rubber stoppers (Chemglass CLS-4209-14), flushed with 80/20 mixture of N_2/CO_2, and autoclaved. Stock peat media tubes were then anaerobically inoculated with 0.05 ml of liquid from enrichments (avoiding peat) that were identified as high methane producers (#s 3, 4, 6, 8, 9, 15, 16, 23, 28; Table 3). Anaerobically prepared and filter sterilized/ autoclaved amendments (Rifampicin, Ampicillin, MES at a 6 or 6.75 pH, TiNta, Vitamins, Yeast Extract, CoM; SI Table 5) were added (0.05 ml/5 ml of peat media) anaerobically outside of the glovebox, appropriate carbon substrates were added (e.g. acetate) and tubes requiring H_2 in the headspace were over pressured. Tubes were again monitored for methane production and visible growth.

4.3.7 Sequencing

Following a successful enrichment and transfer to media, DNA was extracted from the previous enrichment tube or from the original source tube, and PCR confirmed the presence of the *mcrA* and/or 16S bacterial genes (as described above). DNA was then sent for either Sanger sequencing at (genome Quebec), or for amplicon sequencing using the Illumina MiSeq platform (Metagenom Bio Inc.). Sanger sequencing was preformed on the *mcrA* specific primer set

(above) and the 27F-1492 R primer set and trace files were cleaned in MEGA version 6.06 (Tamura et al., 2007) before being compared with known sequences on the NCBI database using BLASTn (Leinonen et al., 2011). Illumina data for methanogens was again generated using the *mcrA* primer set while bacteria were analyzed using the prokaryotic 341F (5' - CCT ACG GGN BGC ASC AG - 3') - 805R (5' - GAC TAC NVG GGT ATC TAA TCC - 3') primer pairing (Sinclair et al., 2015). Output forward and reverse reads were put though a custom pipeline on Ontario's Compute Canada network (www.sharcnet.ca). Briefly, forward and reverse reads were quality filtered, adapter/primers trimmed, and aligned into individual reads using the BBMap package (Bushnell, 2016). QIIME commands were then used to merge data into one FASTA file and then to dereplicate sequences. Sequences were then abundance sorted and singletons discarded using USEARCH8 (Edgar, 2010). Bacterial sequences were clustered at 97% while mcrA sequences were clustered at 85% (Hunger et al., 2011; Yang et al., 2014). Chimeric sequences were removed and taxonomy was assigned to OTUs using a greengenes database for bacteria and a custom *mcrA* library (Yang et al., 2014). Methanogen OTUs were checked to confirm that they were protein coding using Framebot (Wang et al., 2013). Final OTU tables were exported and abundance data compiled and summary plots and statistics done in R version 3.3.1 (R Core Team, 2016).

4.4 Results and Discussion

4.4.1 Enrichment Strategy

Initial enrichments yielded methanogens closely related to those already isolated from peatlands (Bräuer, Cadillo-Quiroz, et al., 2006; Bräuer, Yashiro, et al., 2006; Bräuer et al., 2011). TRFLP

profiles in Figure 4.2 panel A show that the original White River peat community was varied but converged on one dominant organism following enrichment with Rifampicin (antibiotic targeting RNA synthesis primarily in bacteria) and a homopipes buffer (an organic buffer that doesn't appear to supply methanogen substrate precursors; pH 5.4). Methane production was increased 10x in the rifampicin and homopipes sample compared to the 0.76% for the $H_2N_2CO_2$ control. Following TRFLP profiling, both microscopic work (Figure 4.3) and Sanger sequencing of the 16S rRNA gene identified this organism as being 98% similar to *M. boonei*, the organism isolated and described in Bräuer et al. (2011). Most likely this is due to using similar methodology and conditions to enrich the peat and indeed it was shown that post isolation, obtaining the same organism again was relatively easy a second time when using the predetermined conditions and growth substrates (Bräuer et al., 2004; Bräuer, Yashiro, et al., 2006). Thus, our results indicate that the same organism can be isolated under similar conditions from distant peatlands (i.e. Sudbury, ON and Ithaca, NY). Taking these factors into consideration, we shifted focus with later enrichments and attempted to isolate not only hydrogenotrophic methanogens but also acetoclastic and/or diverse metabolic methanogens with abilities to grow on varied carbon substrates (i.e. Methanosarcinales; see Liu, 2010). With this in mind, the ca. 100 bp peak in panels C and D of Figure 4.2 indicated likely presence of acetoclastic methanogens, and peat from these sites became the focus of later enrichments.

On December 7, 2014 we used peat from both Clearwater and Cartier Forest (Figure 4.2, panels C and D) to attempt to enrich and isolate a novel rice cluster II (RCII) species of methanogens (Großkopf et al., 1998). Enrichment treatments are outlined in Tables 4.1 and 4.2 and are the product of numerous shorter trial enrichments (data not shown) using similar substrates and

amendments (concentrations are listed in SI Table 5). Isolation of methanogens has historically focused on environments that have one type of extreme condition. For example temperature extremes (ca. 55-57 °C) have been used to isolate novel methanogens from both natural environments (Harris et al., 1984) as well as in artificial settings such as anaerobic digesters (Kamagata and Mikami, 1991). This strategy has been relatively successful to date; however, enrichment and isolation from environments with more standard conditions are less common (Narihiro and Kamagata, 2013). In this study, we used low temperature (6 °C) to mimic conditions in most northern latitude peatlands and to help target novel species. Duration of enrichments became a factor and substantial amounts of methane (>5%) normally obtained in other enrichment trials were not seen at any point in the incubation. Additionally, it took nearly two years to accumulate samples with >2% methane concentrations. This lengthy process has been shown in other enrichment studies (Vartoukian et al., 2010) and highlights the difficulty in isolating novel organisms. In contrast the second set of enrichments ("Daisy Lake") discussed in this study began nearly a year later and only took two months to reach headspace methane values far in excess of 5% at an incubation temperature of 22 °C (Table 4.3). In this case we took advantage of a peatland that has historically been impacted by smelting activities and subsequent sulfur and metal contamination (see Chapter 3). This allowed us to enrich this peat at a higher temperature and at a higher pH than conditions that led to re-obtaining *M. boonei* (6.75 MES buffer compared to 5.4 homopipes) in other enrichment attempts.

4.4.2 Methanogen Communities

Results of enrichments indicate that there is a strong preference for the presence of hydrogen in the headspace gas mixture with few samples reaching above 2% methane concentrations when

only $N_2 + CO_2$ gas was available (Tables 4.1-4.3). In theory, the absence of hydrogen should lead to the acetoclastic methanogens (Kamagata and Mikami, 1991; Ma et al., 2006; Mori et al., 2012), but these enrichments proved to be the most difficult. We noticed no difference in sequence counts for samples sequenced from the enrichment media itself (Cartier Forest, Clearwater) and samples that were sequenced from the first transfer to artificial media (Daisy Lake samples); *mcrA* reads averaged ca. 4,450 per sample. Any difference in initial abundance of DNA due to dilution during transfer appears to have been overcome by PCR steps prior to sequencing. It is still advisable that during transfer to artificial media a portion of the sample is retained and frozen for later DNA based work as the large number of samples likely means that routine sequencing at every step is often not practical or financially feasible. The methanogens obtained in this work, to our knowledge, are uncultured, but have been found in multiple environments using DNA extraction and marker-gene sequencing-based community fingerprinting approaches (Table 4.4). In large part, the closest related sequences were also obtained from peatland habitats, indicating that these methanogens may have a broad global distribution.

In total, 10 distinct methanogenic strains across 5 of the 7 methanogenic orders were enriched in 15 different enrichment tubes. Of the most abundant sequences, OTU 1 and 3 appear to be hydrogenotrophic methanogens in the Methanomicrobiales, related to *M. boonei*, but likely represent distinct species or strains (Figure 4.4). Franchini and Zeyer (2012) recovered a clone related to the "fen cluster" that formed a distinct OTU in their study from an alpine fen in Oberaar, Switzerland related closely to our OTU 1. This sequence was most common and dominant in Daisy Lake samples (pH 5.1), labeled as follows (culture number-pH): 8-6, 15-6.75,

and 23-6.75, which were all amended with trimethylamine (TMA) and Clearwater 27 that only had CoM added. Given our data, it appears that the relatively culturable strains of this organism from these sites grew well at a high pH, and that TMA is a potential energy source. It should be noted that *M. boonei* was isolated from (Bräuer et al., 2011) and has been found to be abundant in low pH sites (Hales et al., 1996). OTU 1 appeared in samples that had either H_2 or TMA present, and demonstrated in highest relative abundance and highest methane production (7.67%) in enrichments containing the combination of the two (Daisy Lake 15-6.75; Table 4.3). A close phylogenetic relative of OTU1, OTU 3 was the most abundant sequence in four of the samples, however it was only dominant in Clearwater 30 (pH 3.7) and moderately so in Daisy Lake 23, with high rates of co-occurrence of OTU 1 in samples. OTU 3 was abundant in enrichments that had varied substrate additions (methanol, TMA, acetate, and peat media) but it is unclear if these served as substrates for bacterial growth and H_2 production or methanogen growth. Given the phylogenetic positioning (Figure 4.4) it appears that this is a hydrogenotrophic methanogen. The most closely related clone (LN716320) to OTU 3 was not abundant in Hunger et al. (2015), but the most abundant clone in their study (LN716347) was also most closely related to *M. boonei* and was found in two bogs (pH 4.9 and 3.9) and an acidic fen (pH 4.3) in Germany but was absent from a neutral (pH 7.6) fen in the same study. Similarly, Hunger et al. (2015) found that substrate additions of glucose, acetate, and H_2 produced high rates of methane production. Taken together, it appears that OTU 3 is likely a closely related strain of *M. boonei* that also grows at low pH, while OTU 1 is more likely a distinct species that could grow at higher pH optima more commonly found in fens.

There was evidence of non-hydrogenotrophic OTUs dominating four of our enrichments. OTU 2 was most abundant in Daisy Lake 6 but was abundant and dominant in Daisy Lake 9. Not only did OTU 2 cluster within the Methanosarcinales (Figure 4.4), but it also grew well in the presence of acetate providing additional support that hydrogenotrophy was not the primary energy source. A closely related clone (99% ID across 426 nucleotides) was found in a Japanese bog where *Methanosaeta* spp. only accounted for 4.9% of the *mcrA* community which was dominated by *Methanomicrobiales* (Narihiro et al., 2011). OTU 4 was also a dominant sequence, although read numbers were low and sometimes OTUs 1 and 3 were also abundant (e.g., Daisy Lake 16-6.75). Daisy Lake 8-6.75 contained a large proportion of OTU 4 however; it appears that there was a high rate of bacterial growth in this enrichment due to the high read count and overwhelming dominance in the bacterial sequences for this sample. Phylogenetic resolution is a bit unclear as it does not cluster well with either the *Methanosarcina* or *Methanosaeta* and may in fact be related to the *Methanocellas* and RCI lineage which is a hydrogenotrophic methanogen (Sakai et al., 2008).

4.4.3 Dynamics of the Putative Methanogen Syntrophs

Bacterial communities were not the main focus of this work, however when transferring to artificial media it became apparent that anaerobic bacterial growth was easier to obtain than methanogens. Herein, we will focus on three enrichments that had dominant and abundant methanogen communities and their associated bacterial communities. Daisy Lake 9 contained one dominant bacterial OTU (4) most closely related to *Clostridium magnum*, a common soil anaerobic species that produces acetate in the presence of H_2 (Bomar et al., 1991) and in some cases is resistant to ampicillin (Brook et al., 2013). Interestingly, this enrichment did have H_2

present as well as acetate and the methanogen OTU 2 that dominated this enrichment clustered well with known acetoclastic methanogens, indicating a potential mutualistic relationship where if the methanogen is not also hydrogenotrophic, *C. magnum* benefits from the presence of H_2 and the methanogen from the production of acetate. Of the other methanogen enrichments Daisy Lake 23-6.75 also had a dominant (>900 reads) bacterial affiliate (Table 4.5). In this case, the associated bacteria were in OTU 3, closely related to the proposed *Desulfonatronum parangueonese* spp. nov. (Perez-Bernal et al., 2017). This species is anaerobic and known to grow on lactate, formate, pyruvate and ethanol in the presence of sulfate and to produce acetate. This is also initial evidence that sulfate reducers may in fact serve competitive and symbiotic roles with different groups of methanogens. The dominant methanogen in this enrichment was likely hydrogenotrophic (methanogen OTU 1), so there is little evidence of a direct link between the two species. However, one species of *Methanoregula, M. formicica* can grow on formate (Yashiro et al., 2011), so it's possible that this strain of *D. paraangueonese* may also ferment formate as well as acetate. Finally, the Daisy Lake 15-6.75 enrichment had a co-culture of bacteria, both belonging to *Clostridium* (Table 4.5). The more abundant of the two was again the *Clostridium magnum* strain also seen in Daisy Lake 9, and the other abundant species was related to a *Clostridium aciditolerans* strain isolated from a constructed wetland (Lee et al., 2007). The *C. aciditolerans* strain is obligately anaerobic, grows on a variety of substrates, and is known to produce acetate, butyrate and ethanol from glucose (Lee et al., 2007). The strain is not known to use H_2 and subsequently, in contrast to the Daisy Lake 9 sample where an acetoclastic methanogen dominated, the hydrogenotrophic methanogen OTU 1 was dominant. The presence of *Clostridium spp.* in the enrichments in also interesting as the strains present are known to form endospores (Chin et al., 1998; Lee et al., 2007) making them ideal for surviving harsh

environmental conditions such as those in peatlands for long periods of time; a trait common in soil environments (Mandic-Mulec and Prosser, 2011).

4.4.4 Symbiotic Relationships

Methanogenic archaea were enriched with one or more specific bacteria, suggesting a strong interrelationship between these organisms. Syntrophic associations between methanogens and bacteria are well represented in the literature (Mcinerney et al., 2009; Sieber et al., 2010) and appear to be widespread in the environment (Chin et al., 1998; Morris et al., 2013; Kouzuma et al., 2015). It is clear given our data that enrichments with bacterial and methanogens present are far easier to obtain than subsequent attempts to separate the organisms. Methods such as physical separation, use of antibiotics, and a variety of other strategies have been proposed and attempted with limited success (Vartoukian et al., 2010; Pham and Kim, 2012). For example, a novel isolate *Methanocella paludicola* (Sakai et al., 2008) was first enriched and co-cultured with *Syntrophobacter fumaroxidans*, before going through a series of serial dilutions (lasting a year) to eventually obtain the pure methanogen isolate (Sakai et al., 2007). In this case Sakai et al (2007) used *S. fumaroxidans* as a source of H_2 for the methanogen, finding that overpressure in the headspace lead to other already isolated methanogens to outcompete *M. paludicola* for the substrate. Another approach may be the use of a chemostat to both enrich and isolate, from co-culture, methanogens such as acetoclastic ones that require slow but constant additions of growth substrates that are often supplied through symbiotic relationships. It is obvious that methanogen isolation is difficult and instead of focusing purely on isolation, we propose using molecular- and/or genetic-based tools and co-culturing as a realistic method of obtaining valuable information such as substrate limitations or temperature optima from novel species in

enrichments. If mutualistic, syntrophic relationships are as frequent in methanogens as evidenced here, then the central dogma surrounding methane production needs to shift to include bacterial interactions. Future research should consider not what the methanogens do and need, but rather what the co-culture or mixed culture does and needs, and how the organisms interact and function as a whole.

4.4.5 Future Directions

Looking forward there is an obvious need for expanding sequence libraries beyond that of short sequence reads such as clones and amplicon sequences from platforms like MiSeq. Longer sequence reads will be critical in developing a fundamental understanding of methanogen diversity and functioning in ecosystems across the globe and will aide in obtaining a more complete picture of the controls over GHG emissions and the role methanogens play, and technologies are improving in this area (see Rhoads and Au 2015). Complementary to this is the need for baseline physiological studies on these organisms that aide in identifying basic traits such as optimal growth conditions, substrate utilization, and potential mutualistic/symbiotic relationships. There is no perfect way forward, however we believe that there is a middle ground between pure culturing and genetic based approaches that will provide useful information moving forward. We propose that future research should focus on enriching for and obtaining novel organisms that can be maintained in non-pure cultures. With current technologies, researchers are able to then get whole genome sequences from shotgun sequencing approaches (Mondav et al., 2014), as well as run complementary physiological studies to determine basic growth parameters. This information can then be used to look “backwards” and compare the whole genome against the databases of clones and amplicon reads to determine global or local

relevance of a particular organism (Harbison et al., 2016). While enrichment work does not meet IJSEM standards for culturing, it does provide far more information than is currently available with clone and amplicon sequences that are abundant in sequence databases. Furthermore, by obtaining whole genomes, genetic potential via gene analysis can be determined to further aid attempts to isolate organisms should that become an eventual end goal. This need for quality long DNA reads is not unique to the archaeal or methanogen world, but environmental microbiology in general could adopt these concepts to move the field forward as a whole and obtain critical information from the "99% of unculturable" microorganisms (Pham and Kim, 2012).

4.5 Conclusions

Our work indicates that novel methanogens from at least five of the seven methanogenic orders are prevalent in four peatland ecosystems around Sudbury, ON. Given the importance of these sites in global greenhouse gas production, work to identify these organisms is of critical importance. While isolation of novel methanogens has proven to be difficult, both here and elsewhere, by combining classical isolation and enrichment techniques with modern sequencing technologies, we were able to obtain a reasonable amount of information to describe novel organisms. This approach will help to bolster the overall number of sequences in our reference libraries, while providing a broad sense of potential growth conditions and methanogen phylogeny. Obtaining novel sequences from peatlands is of utmost importance as few relevant methanogenic isolates have been cultured from these habitats (Cadillo-Quiroz et al., 2009, 2014; Bräuer et al., 2011), and our overall understanding of peatland methanogens is largely dependent on work done in other ecosystems. Novel methanogens in peatlands, and other habitats, will

likely be found in areas with at least one unique "defining" characteristic (selective pressure) such as high metal concentration or a pH extreme that allows for a unique approach in enrichment work. That is to say directly replicating past work will likely not lead to discovery of novel organisms. Additionally, future work should attempt to go beyond amplicon sequencing and incorporate shotgun metagenomic approaches in an attempt to obtain whole genomes of both methanogens as well as their potential syntrophic bacterial partners. While work in obtaining isolates is still viewed as important and should be attempted, this should not be the sole goal in the modern age of sequencing technology.

4.6 Tables and Figures

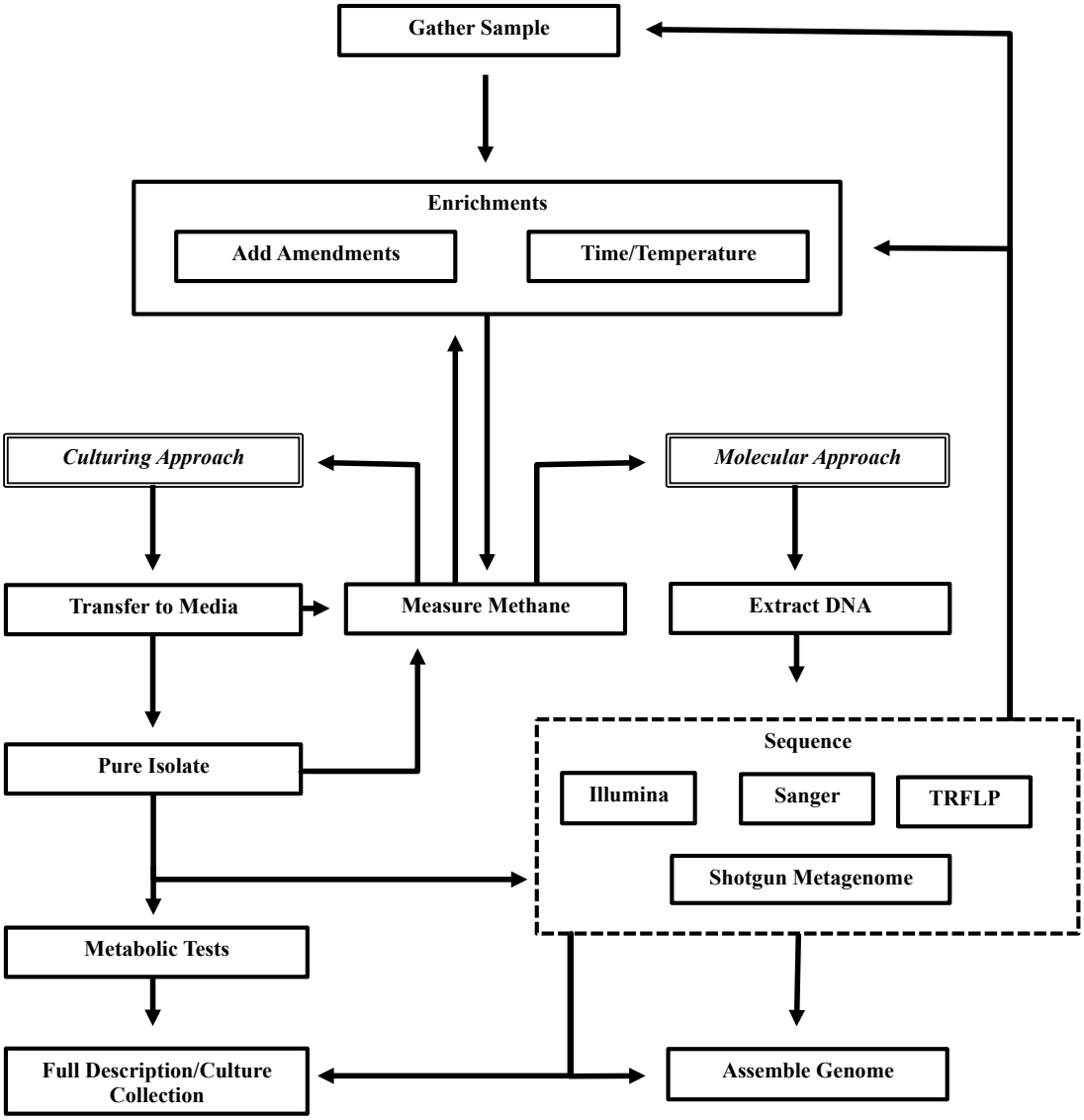

Figure 4.1 Schematic of enrichment methods. Traditional culturing methods follow the pathway on the left where after successful enrichment cultures are grown on artificial media. The right side of the figure depicts the modern approach where successful enrichments are sequenced and enrichment condition information is paired with modern sequencing analysis.

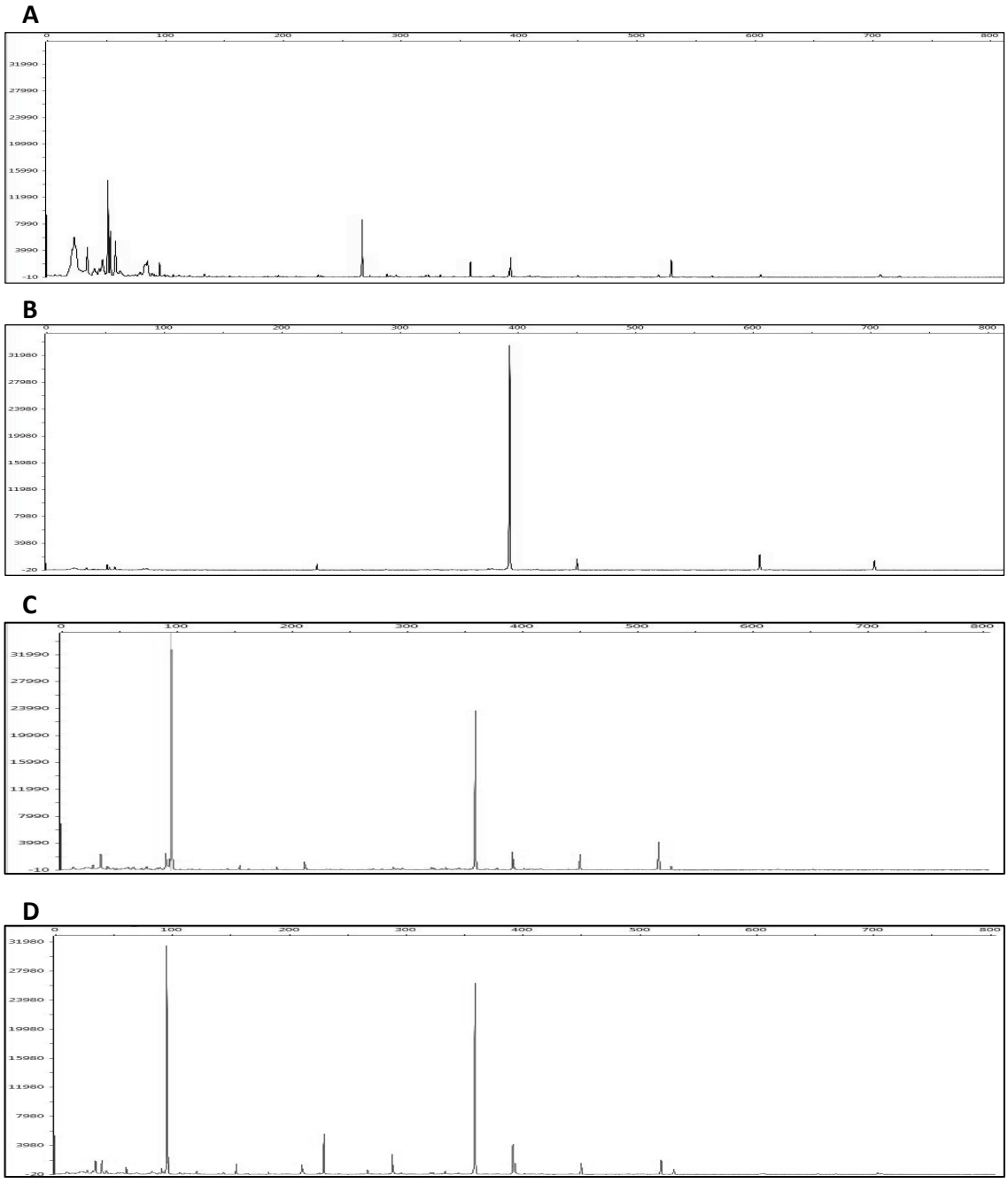

Figure 4.2 TRFLP electropherograms. Archaeal SSU rRNA genes amplified with 1AF-1100R-HEX and restricted with HhaI and Sau96I using DNA extracts of from (A) White River Fen peat, (B) a 30 day anaerobic enrichment of White River Fen peat in a 9:1 (water:peat) mix amended with 0.1ml of homopipes buffer (pH 5.4) and 0.1 mg of rifampicin, (C) Clear Water peat, and (D) Cartier Forest peat.

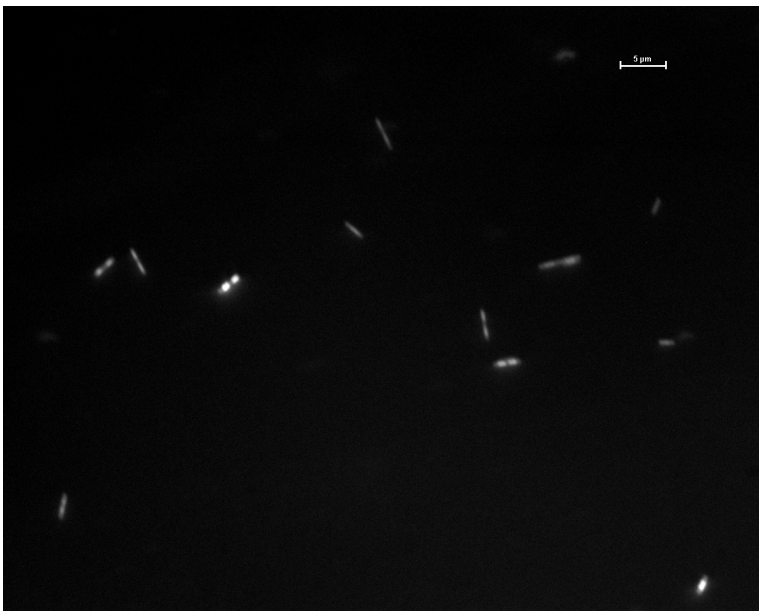

Figure 4.3 Epiflorescence image of cells from White River Fen peat enriched anaerobically with homopipes buffer (pH 5.4) viewed at 1000x, stained with acridine orange (white bar = 5 µm).

Table 4.1 Cartier forest enrichments. Experimental layout for enrichment samples of Cartier Forest peat incubated for over two years at 6 °C. Additions consisted of 0.1 mL into a 9:1 water to peat mixture following concentrations in SI Table 5. Methane was measured in all samples on three dates.

Cartier Forest					
Sample	Headspace	Additions	% CH_4 May 26, 2015	% CH_4 October 15, 2015	% CH_4 July 24, 2017
1	N_2CO_2		0.07	0.81	1.12
2	N_2CO_2	Rifampicin	0.00	0.34	0.38
3	N_2CO_2	Ampicillin	0.07	0.03	0.13
4	N_2CO_2	Ethanol	0.14	0.08	1.31
5	N_2CO_2	Acetate	0.25	0.12	0.74
6	N_2CO_2	Low Sulfide	0.10	0.04	0.10
7	N_2CO_2	Sulfide	0.04	0.02	0.12
8	N_2CO_2	Homopipes	0.04	0.02	0.16
9	N_2CO_2	TinTA	0.06	0.01	0.19
10	N_2CO_2	CoM	0.01	0.00	0.02
11	N_2CO_2	Vitamins	0.00	0.00	0.01
12	N_2CO_2	Butanoate	0.03	0.02	0.05
13	N_2CO_2	Propionate	0.00	0.00	0.00
14	N_2CO_2	Glucose	0.01	0.01	0.04
15	N_2CO_2	MES	0.06	0.04	0.10
16	$H_2N_2CO_2$		0.08	0.06	0.39
17	$H_2N_2CO_2$	Rifampicin	0.01	0.01	0.11
18	$H_2N_2CO_2$	Rifampicin Ethanol	0.01	0.01	0.07
19	$H_2N_2CO_2$	Rifampicin Acetate	0.01	0.01	0.14
20	$H_2N_2CO_2$	Rifampicin CoM	0.00	0.00	0.01
21	$H_2N_2CO_2$	Rifampicin Sulfide	0.00	0.00	0.06
22	$H_2N_2CO_2$	Rifampicin Homopipes	0.01	0.01	0.03
23	$H_2N_2CO_2$	Rifampicin PM1	0.00	0.00	0.02
24	$H_2N_2CO_2$	Ampicillin	0.04	0.02	0.36
25	$H_2N_2CO_2$	Ampicillin Ethanol	0.05	0.03	0.06
26	$H_2N_2CO_2$	Ampicillin Acetate	0.12	0.08	1.25
27	$H_2N_2CO_2$	Ampicillin CoM	0.08	0.04	0.15
28	$H_2N_2CO_2$	Ampicillin Sulfide	0.01	0.00	0.02
29	$H_2N_2CO_2$	Ampicillin Homopipes	0.04	0.04	0.56
30	$H_2N_2CO_2$	Ampicillin PM1	2.02	1.33	4.54
Started Decemper 7, 2014		Incubated at 6 °C			

Table 4.2 Clear water enrichments Experimental layout for enrichments samples of Clear Water peat incubated for over two years at 6 °C. Additions consisted of 0.1 mL into a 9:1 water to peat mixture following concentrations in SI Table 5. Methane was measured in all samples on three dates.

Clear Water						
Sample	Headspace	Additions		% CH_4 May 26, 2015	% CH_4 October 15, 2015	% CH_4 July 24, 2017
1	N_2CO_2			0.00	0.00	0.01
2	N_2CO_2	Rifampicin		0.00	0.00	0.00
3	N_2CO_2	Ampicillin		0.00	0.00	0.00
4	N_2CO_2	Ethanol		0.01	0.00	0.05
5	N_2CO_2	Acetate		0.00	0.00	0.01
6	N_2CO_2	Low Sulfide		0.00	0.00	0.01
7	N_2CO_2	Sulfide		0.01	0.00	0.04
8	N_2CO_2	Homopipes		0.00	0.00	0.01
9	N_2CO_2	TinTA		0.00	0.00	0.00
10	N_2CO_2	CoM		0.01	0.00	0.01
11	N_2CO_2	Vitamins		0.00	0.00	0.00
12	N_2CO_2	Butanoate		0.00	0.01	0.04
13	N_2CO_2	Propionate		0.00	0.00	0.01
14	N_2CO_2	Glucose		0.00	0.00	0.03
15	N_2CO_2	MES		0.00	0.00	0.00
16	$H_2N_2CO_2$			0.04	0.05	0.32
17	$H_2N_2CO_2$	Rifampicin		0.03	0.04	0.09
18	$H_2N_2CO_2$	Rifampicin	Ethanol	0.02	0.04	0.22
19	$H_2N_2CO_2$	Rifampicin	Acetate	0.02	0.02	0.18
20	$H_2N_2CO_2$	Rifampicin	CoM	0.01	0.01	0.10
21	$H_2N_2CO_2$	Rifampicin	Sulfide	0.01	0.01	0.14
22	$H_2N_2CO_2$	Rifampicin	Homopipes	0.01	0.01	0.04
23	$H_2N_2CO_2$	Rifampicin	PM1	0.01	0.01	0.19
24	$H_2N_2CO_2$	Ampicillin		0.80	0.09	0.29
25	$H_2N_2CO_2$	Ampicillin	Ethanol	0.97	0.14	0.99
26	$H_2N_2CO_2$	Ampicillin	Acetate	1.15	0.26	1.72
27	$H_2N_2CO_2$	Ampicillin	CoM	1.42	0.63	2.04
28	$H_2N_2CO_2$	Ampicillin	Sulfide	0.01	0.01	0.06
29	$H_2N_2CO_2$	Ampicillin	Homopipes	0.15	0.01	0.04
30	$H_2N_2CO_2$	Ampicillin	PM1	1.99	1.15	3.43
Started Decemper 7, 2014			Incubated at 6 °C			

Table 4.3 Daisy lake enrichments Experimental layout for enrichments samples of Daisy Lake peat incubated for two months at 22 °C. Additions consisted of 0.1 mL into a 9:1 water to peat mixture following concentrations in SI Table 5. Select enrichments were transferred to artificial media following successful enrichment.

Daisy Lake					
					% CH_4
Sample	Headspace		Additions		Feb 17, 2016
1	$H_2N_2CO_2$	MES	Rifampicin	Methanol	4.84
2	$H_2N_2CO_2$	MES	Rifampicin	CoM	3.69
3	$H_2N_2CO_2$	MES	Rifampicin	TMA	5.55
4	$H_2N_2CO_2$	MES	Rifampicin	Acetate	4.56
5	$H_2N_2CO_2$	MES	Rifampicin	Vitamins	6.15
6	$H_2N_2CO_2$	MES	Ampicillin	Methanol	8.51
7	$H_2N_2CO_2$	MES	Ampicillin	CoM	0.75
8	$H_2N_2CO_2$	MES	Ampicillin	TMA	7.34
9	$H_2N_2CO_2$	MES	Ampicillin	Acetate	7.85
10	$H_2N_2CO_2$	MES	Ampicillin	Vitamins	7.62
11	$H_2N_2CO_2$	MES	Ampicillin	PM1	0.66
12	$H_2N_2CO_2$	MES	Rifampicin	PM1	3.88
13	$H_2N_2CO_2$			Methanol	2.01
14	$H_2N_2CO_2$			CoM	4.86
15	$H_2N_2CO_2$			TMA	7.67
16	$H_2N_2CO_2$			Acetate	8.46
17	$H_2N_2CO_2$			Vitamins	8.14
18	$H_2N_2CO_2$			MES	8.44
19	$H_2N_2CO_2$			Rifampicin	3.96
20	$H_2N_2CO_2$			Ampicillin	4.96
21	N_2CO_2	MES	Rifampicin	Methanol	1.55
22	N_2CO_2	MES	Rifampicin	CoM	0.02
23	N_2CO_2	MES	Rifampicin	TMA	2.44
24	N_2CO_2	MES	Rifampicin	Acetate	0.42
25	N_2CO_2	MES	Rifampicin	Vitamins	0.07
26	N_2CO_2	MES	Ampicillin	Methanol	1.62
27	N_2CO_2	MES	Ampicillin	CoM	0.04
28	N_2CO_2	MES	Ampicillin	TMA	2.35
29	N_2CO_2	MES	Ampicillin	Acetate	0.56
30	N_2CO_2	MES	Ampicillin	Vitamins	0.03
31	N_2CO_2	MES	Ampicillin	PM1	0.01
32	N_2CO_2	MES	Rifampicin	PM1	0.03
33	N_2CO_2			Methanol	1.25
34	N_2CO_2			CoM	0.01
35	N_2CO_2			TMA	2.70
36	N_2CO_2			Acetate	0.39
37	N_2CO_2			Vitamins	0.01
38	N_2CO_2			MES	0.01
39	N_2CO_2			Rifampicin	0.03
40	N_2CO_2			Ampicillin	0.01
Started Decemper 14, 2015			Incubated at 22 °C		

Table 4.4 Methanogen OTUs. Amplicon counts from Illumina sequencing of the *mcrA* gene. Of 28 total OTUs detected across 100 enrichment samples, only 10 OTUs had total read counts over 200; less abundant OTUs were excluded from analysis. Sample name numbers indicate treatments listed in Tables 1-3 and MES buffer pH (Cartier and Clear Water samples were unbuffered).

OTU	Daisy #6 MES 6	Daisy #6 MES 6.75	Daisy #8 MES 6	Daisy #8 MES 6.75	Daisy #9 MES 6	Daisy #15 MES 6	Daisy #15 MES 6.75	Daisy #16 MES 6	Daisy #16 MES 6.75	Daisy #23 MES 6	Daisy #23 MES 6.75	Cartier Forest #30	Clear Water #26	Clear Water #27	Clear Water #30	Coverage (%)	Identity (%)	Closest Clone	Accession Number	Citation
1	1753	892	2225	14	475	1264	9526	2529	467	1088	3996	1592	1989	2718	1004	100	96	OA_E11	HE774285	Franchini and Zeyer, 2012
2	0	956	0	1	4304	24	0	1593	0	13	719	978	0	556	1368	100	99	SpM13	AB570067	Narihiro, et al., 2011
3	1915	74	1114	69	828	717	306	276	722	2818	384	230	2673	184	4313	100	99	GKC12	LN716320	Hunger, et al., 2015
4	31	1	16	908	69	5	849	14	724	33	3	18	24	1	5	100	100	BogVII	AJ586247	Galand, et al., 2005
5	0	280	0	0	1	0	0	695	0	0	74	309	0	338	2	100	98	SpM9	AB570063	Narihiro, et al., 2011
6	0	9	0	1	73	7	6	159	8	8	279	198	1	107	6	100	97	329_19g	KT225456	Söllinger, et al., 2016
7	119	1	41	19	1	10	3	1	105	405	0	1	61	0	11	99	97	Kd3	LT632529	*Unpublished*
8	0	2	0	1	16	22	0	61	0	365	42	19	0	23	26	100	99	OA_B01	HE774271	Franchini and Zeyer, 2012
9	38	3	21	9	18	11	17	21	81	187	14	24	31	10	83	99	100	Ac32	LT632444	*Unpublished*
10	0	6	2	0	0	1	4	172	5	44	213	76	8	86	0	100	99	A21F12	LN716961	Hunger, et al., 2015

Table 4.5 Bacterial OTUs. Amplicon counts from Illumina sequencing of the 16S rRNA gene. Only OTUs with total read counts over 1000 were included in analysis, excluding 69 of the total 78 OTUs across 100 enrichment samples. Removed OTUs were never a dominant component of the community composition. Sample name numbers indicate treatments listed in Tables 1-3 and MES buffer pH (Cartier and Clear Water samples were unbuffered).

OTU	Daisy #6 MES 6	Daisy #6 MES 6.75	Daisy #8 MES 6	Daisy #8 MES 6.75	Daisy #9 MES 6	Daisy #15 MES 6	Daisy #15 MES 6.75	Daisy #16 MES 6	Daisy #16 MES 6.75	Daisy #23 MES 6	Daisy #23 MES 6.75	Cartier Forest #30	Clear Water #26	Clear Water #27	Clear Water #30	Coverage (%)	Identity (%)	Closest Published Bacteria	Accession Number	Citation
1	24	326	2095	15179	25	0	0	83	68	0	0	0	0	0	0	100	96	Paludibacter propionicigenes	CP002345	Gronow, et al., 2011
2	0	0	0	0	0	0	0	0	0	0	0	1827	3	9439	0	100	100	clone A14E04	LN715329	Hunger, et al., 2015
3	0	0	0	0	0	0	0	0	0	4195	3713	0	0	0	0	100	95	Desulfonatronum sp. strain PAR190	KY041866	Pérez-Bernal, et al., 2017
4	833	0	553	170	11250	4909	3591	1074	532	0	2	0	0	0	0	100	99	Clostridium magnum strain 120flt5	MG648150	*Unpublished*
5	13	0	3	4	39	104	82	3675	2396	0	0	0	0	0	0	100	98	Clostridium sp. strain RPec1	Y15985	Chin, et al., 1998
6	0	0	0	0	0	0	0	0	0	0	0	0	3875	0	199	100	99	Bacterium K-5b10	AF524859	Sizova, et al., 2003
7	0	0	0	0	0	0	0	0	0	0	0	0	0	96	1911	100	99	Rhodospirillaceae bacterium IA_FO_10	LN831188	Labadie, et al., 2015
8	526	0	573	195	312	3030	2078	6	1	0	0	0	0	0	0	100	98	Clostridium aciditolerans strain JW/YJL-B3	NR_043557	Lee, et al., 2007
9	9	0	0	0	0	0	0	0	4	568	559	0	0	0	0	100	98	Desulfovibrio sp. LG-2009	FN557161	*Unpublished*

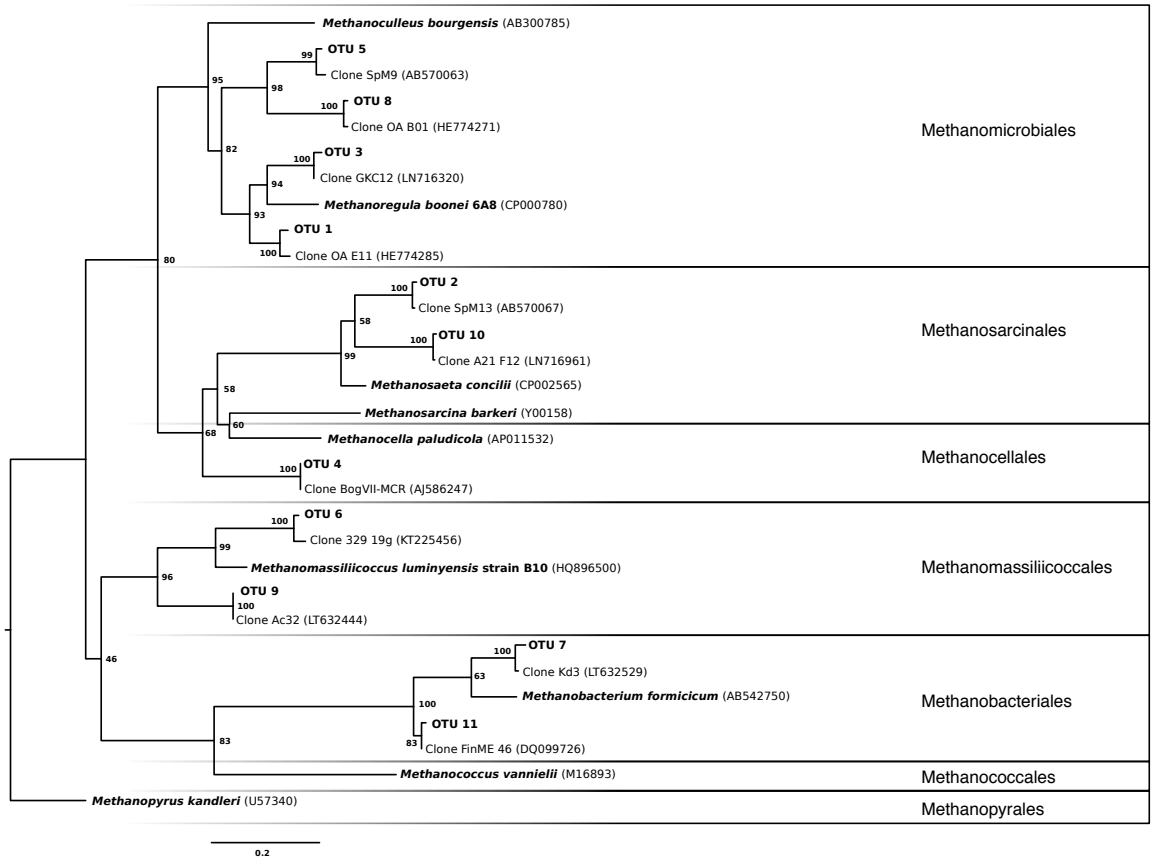

Figure 4.4 Phylogenetic tree of *mcrA* gene. Maximum likelihood tree of enrichments from Daisy Lake, Cartier Forest, and Clear Water. A Bayesian model with 1000 replicates was used and node labels indicate bootstrap values. Branches are arranged by methanogen orders according to cultured representatives. The tree was rooted to *Methanopyrus kandleri*. Clones represent the closest published match by BLAST search.

Chapter 5 Conclusions

5.1 Summary

This thesis builds on an extensive history of peatland methane dynamics work, contributing several novel conclusions to the field. Broadly speaking, peatlands are large C sinks and are undergoing shifts in abiotic conditions with a changing climate and other anthropogenic stressors. These shifts in abiotic factors will likely cause dramatic changes, especially in northern peatlands where permafrost and other stable forms of peat are expected to undergo a state change in the coming years. It is therefore important to understand the controls on methane production at a variety of scales ranging from global rates of production to physiological ecology of individual species of methanogens. The overall goals of this work were to i) determine if broad controls on methane production exist at a global scale, ii) evaluate methanogen community structure and methane production rates across a gradient of historic and contemporary metal and sulfur deposition, and iii) develop a novel strategy of obtaining new methanogens and bacterial symbionts through enrichment and culturing techniques. In large part goals were met on these fronts and they help close existing knowledge gaps regarding peatlands and methane production.

5.2 General Conclusions

Prior to this work, analysis of methane potential production on a global scale was limited to literature reviews, and for the first time we present an analysis of global methane production using a standardized biochamber/laboratory methodology. From this work we were able to determine the relationship between major factors influencing methane production (rising above site-specific controls and variability), including pH, vegetation composition, moisture content, and peat chemistry. It appeared that sites with higher pH and more towards the rich fen spectrum

of peatlands with vascular plant cover are more conducive to methanogenesis. This global-scale pattern is consistent with similar conclusions that have been drawn from individual studies, and strongly highlights concerns of anthropogenic stressors (Dieleman et al., 2015) and atmospheric pollution deposition (Larmola et al., 2013) that can shift bog and poor fen vegetation to rich fen-like communities in a matter of a few years.

There is an important gap in knowledge surrounding the microorganisms responsible for methane production in peatlands. Methanogens as a whole are largely underrepresented in culture collections, partially due to the relative difficulty in isolating and culturing anaerobic, metabolically limited, and slow growing microorganisms. The situation is more extreme at present for acidophilic methanogens (noting that most peatlands are moderately to highly acidic), with only the first strain isolated in 2006 and formally described in 2011 (Bräuer et al., 2006; Bräuer et al., 2011). However, it is shown here that there are approaches for elucidating methanogen physiological ecology may be to strive for mixed enrichment cultures using novel approaches (substrates and buffers) that are closely monitored and guided by contemporary DNA-based fingerprinting techniques. We showed that using past enrichment conditions only led to obtaining previously cultured organisms. It is thus clear that in order to obtain novel methanogens (or perhaps microorganism in general) there must be a distinctly different condition in the process; a unique inoculum alone does not work. That said, site selection and inoculum source is still important for enriching novel organisms, and the Sudbury peatland sites provided an area rich in novel methanogens due likely to their history of high metal and S contamination. Not only did we show that these sites had largely unknown methanogen communities in comparison to regional reference sites, but subsequent attempts to enrich for these organisms *in*

vitro proved to be successful and novel members from the majority of the methanogen families, along with their growth conditions and bacterial syntrophic symbionts, were uncovered. Of particular note is the need to cast a broad net in order to identify novel organisms, and by using rapid sequencing/fingerprinting technologies the enrichment strategies and attempts to isolate can be more rapidly iterated; increasing the chance of obtaining novel genomes from culturing methodologies that are exceptionally slow and prone to growing previously cultured organisms.

5.3 Future Directions

Laboratory incubations are a commonplace necessity in peatland C cycling research, given the virtual impossibility of partitioning gross process rates from net fluxes in situ. These are widely accepted as a valid method for determining rates of methane production in a given site, as evidenced by the ongoing number of publications relying on them. Cross-study comparisons are tenuous however. By using unrealistic temperatures, variable incubation durations, different sampling and storage methods, etc., results can be confounded and artificially inflate or reduce the role that methanogens may be playing in a particular system. It was shown here that at a global scale only a few distinct sites were truly high producers of potential methane (normalized for global warming potential and noting that this was the product of high methane production and not low CO_2 production). Furthermore a site may contain areas of high methane production, but it should not be the end goal of an incubation study to obtain high levels of methane as *in situ* conditions might vary dramatically from conditions needed to obtain these values. In the literature, incubation studies focusing on general mineralization rates/CO_2 production are typically short in contrast to methane production potentials. In the global incubation study, we found that CO_2 production rates are in fact higher than methane production rates and when

accounting for GWP contribute comparably, indicating that under incubation conditions, both GHGs are important contributors to climate change.

After water table position, should a researcher or land manager only measure one simple parameter in a peatland ecosystem with regards to predicting methane production and methanogen community it should be pH. However, the role plant communities play in peatlands and methane emissions are substantial and often controlled by interactions of pH and water table position. More work need to be done to parse out the specific interactions of plant and microbial communities, nutrient availability, and overall methane production and release (the latter another very important sub-field that this thesis did not address). In terms of other relevant peat chemical controls, the role of metals in methanogen activity and methane production is still largely unknown, and most of our current understanding comes from environments that are not likely comparable, such as anaerobic digesters. It will be important for future researchers to conduct individual and combined metal additions in more natural settings to begin to start identifying how methane production rates are affected and the dynamic interplay of the microbial communities. It was clear that large amounts of metals and sulfur (and/or the interactive effects of vegetation changes) caused dramatic shifts in methanogen community structure and methane production, but just as likely is that specific combinations of metals at more biologically relevant concentrations would increase methane production.

The modern era of DNA fingerprinting and omics-approaches have developed rapidly over the past two decades and the field is at a point where DNA sequencing is both rapid and inexpensive. This has become problematic in that reference strain libraries for soils (and especially peat soils)

and other complex environments are virtually always insufficient to tell an investigator anything physiologically or ecologically meaningful about the microorganisms that are now easily detectable based on marker gene sequences retrieved directly from the environment. It is therefore important to consider taking a step back and begin to lean on culturing and enrichment methods more strongly. While it is somewhat impractical to isolate and fully describe a novel species, especially the difficult-to-culture methanogens, researchers should employ enrichment techniques to better understand the physiological and chemical constraints on novel organisms (beyond the methanogens, expanding broadly to bacteria, fungi, and archaea). Further, partial and even full genomes can be obtained from these enrichments. Other valuable information such as potential symbiotic relationships, substrate utilization, and growth optima may also be determined in non-pure enrichments, and given that it is increasingly understood that microbes do not live in isolation in nature, evaluation of growth-condition responses of mixed cultures might better represent in situ controls. With this physiological ecological information in hand, researchers can then use the amplicon sequence archives as a database to determine the novel organism's importance (distribution and abundance) on global scales.

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Supplementary Information

SI Table 1 Total elements means by site n=6 for most samples (Hg all below DL 2 mg/kg). Distances (km) for sites are as follows: DL (6.8), LU (8.2), BL (13.6), CW (20.2), LL (20.5), BR (24.0), AS (26.5), MG (31.0), CL (50.1), CF(50.2).

Site	DL	LU	BL	CW	LL	RC	AS	MG	CL	CF
Ag	NA	NA	NA	NA	1	NA	NA	NA	NA	NA
Al	22333	6672	24050	4367	20700	31913	13733	14215	1542	3882
As	19	6	5	6	6	3	13	8	2	2
B	11	5	5	6	10	7	6	7	6	5
Ba	187	117	174	47	214	274	165	100	24	51
Be	1	0	1	0	1	1	1	1	NA	0
Bi	2	1	4	2	4	1	2	2	0	1
Ca	13820	14988	8237	4060	12145	6602	8412	4662	2182	3408
Cd	4	1	2	1	3	2	2	1	0	1
Co	40	11	13	5	19	6	11	8	1	3
Cr	26	10	20	9	33	28	19	18	4	8
Cu	737	189	509	316	590	99	245	160	21	25
Fe	6697	4808	4037	2058	7993	5427	6157	3437	1139	2280
In	NA	NA	0	NA	0	NA	NA	NA	NA	NA
K	2593	793	1840	464	3174	7130	1982	1677	462	752
Li	12	NA	NA	NA	9	5	3	3	NA	1
Mg	2192	1129	1498	496	2463	1890	1353	848	549	759
Mn	63	54	84	28	129	123	109	51	9	29
Mo	NA	NA	NA	NA	NA	4	NA	NA	NA	NA
Na	1098	450	1005	342	2384	7930	1657	1174	269	539
Ni	2401	410	478	183	694	140	217	127	19	30
P	1437	805	2020	603	1202	1200	1345	1279	417	424
Pb	31	60	47	26	57	31	48	31	16	27
Rb	13	3	8	2	12	24	10	7	2	4
Sb	1	0	0	1	1	1	0	0	0	0
Sc	8	3	11	2	8	6	8	8	1	1
Se	5	3	6	2	7	2	4	3	2	2
Sn	2	72	3	1	4	2	2	3	1	1
Sr	81	51	49	20	94	131	52	38	15	27
Th	5	1	7	1	5	3	3	5	NA	1
Ti	594	215	449	172	818	1222	482	478	77	192
Tl	0	0	0	0	0	0	0	0	0	0
U	4	0	20	0	4	1	2	4	0	0
V	18	8	13	7	30	21	15	16	2	7
W	NA	NA	1	NA	1	1	NA	1	NA	NA
Y	25	3	40	2	17	7	15	24	1	2
Zn	120	32	35	21	78	38	65	31	25	26
Zr	22	7	15	6	25	78	18	23	2	7

SI Table 2 Extractable element means by site n=6 for most samples. Elements below detection limits (mg/kg) include Ag 1, Be 0.05, Bi 0.3, Hg 2, In 0.05, Mo 3, Sn 0.7, Th 0.7, Tl 0.06, W 0.8, Zr 0.5. Distances (km) for sites are as follows: DL (6.8), LU (8.2), BL (13.6), CW (20.2), LL (20.5), BR (24.0), AS (26.5), MG (31.0), CL (50.1), CF(50.2).

Site	DL	LU	BL	CW	LL	RC	AS	MG	CL	CF
Al.Li	26	7	20	10	17	29	9	29	6	11
As.Li	2	1	1	3	1	0	1	1	0	0
B.Li	3	3	3	3	3	2	2	4	6	3
Ba.Li	4	6	6	5	5	7	7	7	13	8
Ca.Li	679	681	497	387	567	167	474	359	291	279
Cd.Li	0	NA	0	0	0	0	0	0	NA	0
Co.Li	1	0	1	0	0	0	0	1	0	0
Cr.Li	NA	NA	NA	NA	NA	NA	NA	NA	0	1
Cu.Li	1	0	1	2	1	0	0	0	NA	NA
Fe.Li	112	87	58	45	82	39	55	75	20	28
K.Li	28	41	50	85	73	55	86	139	190	156
Mg.Li	246	140	161	121	192	61	161	136	161	155
Mn.Li	4	3	7	3	5	6	9	5	1	2
Na.Li	158	185	178	259	172	129	152	222	399	235
Ni.Li	43	6	25	7	8	4	4	3	1	1
P.Li	NA	NA	NA	15	NA	NA	NA	NA	NA	NA
Pb.Li	0	NA	NA	0	0	0	0	0	NA	0
Rb.Li	0	0	0	0	0	0	0	0	1	1
Sb.Li	0	0	0	0	0	0	0	0	0	0
Sc.Li	0	0	0	0	0	0	0	0	0	0
Se.Li	NA	NA	NA	0	0	NA	NA	NA	1	NA
Sr.Li	3	2	2	2	3	2	2	2	2	2
Ti.Li	1	0	0	0	1	NA	0	NA	1	1
U.Li	NA	NA	0	NA	NA	NA	NA	NA	NA	NA
V.Li	0	0	0	0	0	0	0	0	NA	0
Y.Li	0	NA	0	0	0	0	NA	0	NA	NA
Zn.Li	9	6	7	7	7	6	7	9	13	8

SI Table 3 Total element means by site and depth, n=3 for most samples. Only Hg was below detection limits (2 mg/kg). Distances (km) for sites are as follows: DL (6.8), LU (8.2), BL (13.6), CW (20.2), LL (20.5), BR (24.0), AS (26.5), MG (31.0), CL (50.1), CF(50.2).

Site	DL		LU		BL		CW		LL		RC		AS		MG		CL		CF	
	Middle	Deep	Middle	Deep	Middle	Deep	Middle	Deep	Middle	Deep	Middle	Deep	Middle	Deep	Middle	Deep	Middle	Deep	Middle	Deep
Ag	NA	NA	NA	NA	NA	NA	NA	NA	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Al	16867	27800	7630	5713	20633	27467	4387	4347	9800	31600	13360	50467	12333	15133	6197	22233	1733	1350	4167	3597
As	36	2	10	1	8	1	10	1	9	2	5	1	24	1	13	4	3	1	4	1
B	9	12	4	7	5	5	6	7	8	12	5	9	8	5	7	7	6	6	6	4
Ba	215	158	124	110	152	196	64	30	164	264	168	381	173	156	96	103	23	26	62	39
Be	1	1	0	0	1	1	0	NA	1	1	1	1	1	1	0	1	NA	NA	0	0
Bi	2	NA	1	NA	4	NA	2	NA	4	NA	1	NA	2	NA	2	NA	0	NA	1	NA
Ca	11207	16433	9543	20433	2773	13700	4960	3159	9023	15267	2883	10320	6860	9963	4803	4520	1753	2610	4337	2480
Cd	7	1	2	0	3	1	2	0	4	1	2	1	4	1	2	0	1	0	2	0
Co	77	3	18	4	21	5	8	1	33	5	7	4	18	3	13	2	1	1	4	1
Cr	20	32	12	8	18	22	8	9	23	43	11	46	21	16	9	27	5	4	9	7
Cu	1414	60	353	25	960	59	620	12	1098	83	178	20	440	51	167	153	40	3	40	10
Fe	9730	3663	5663	3953	3843	4230	2800	1316	6893	9093	2927	7927	8913	3400	3970	2903	1457	821	3267	1294
In	NA	NA	NA	NA	0	NA	NA	NA	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
K	1847	3340	854	732	1517	2163	671	256	1111	5237	1627	12633	2107	1857	910	2443	751	174	1250	253
Li	5	20	NA	NA	NA	NA	NA	NA	NA	9	2	8	3	3	2	4	NA	NA	1	1
Mg	1780	2603	704	1553	866	2130	519	474	1697	3230	533	3247	1463	1243	812	884	540	557	1133	384
Mn	90	36	67	41	64	104	34	21	124	135	63	182	169	50	69	33	14	4	48	9
Mo	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4	NA	NA	NA	NA	NA	NA	NA	NA
Na	1077	1119	517	383	912	1098	504	180	1010	3757	1427	14433	1850	1463	528	1820	325	214	900	179
Ni	4737	66	776	44	891	64	355	10	1334	54	256	24	409	25	237	16	34	4	53	6
P	1573	1300	1150	460	1913	2127	857	349	1247	1157	1347	1054	1370	1320	1048	1510	513	321	582	266
Pb	56	6	25	95	89	4	51	2	105	9	47	14	90	5	54	8	30	2	51	2
Rb	9	17	3	2	7	9	2	1	5	18	5	42	12	8	4	9	3	1	6	1
Sb	1	0	1	0	1	0	1	NA	1	0	1	0	1	0	0	1	0	NA	0	NA
Sc	5	12	4	2	5	17	3	2	2	13	4	8	3	13	1	16	1	1	1	2
Se	7	3	4	2	8	4	3	1	10	3	2	1	5	3	4	3	2	1	2	1
Sn	2	2	1	143	3	4	1	1	4	3	2	3	2	NA	2	4	1	1	2	1
Sr	71	91	43	59	26	71	26	14	68	121	37	225	45	59	31	44	13	18	37	17
Th	2	7	1	1	3	12	1	2	1	8	1	4	2	5	1	10	NA	NA	2	1
Ti	449	739	245	185	385	514	214	129	417	1220	367	2077	459	505	168	787	93	61	240	145
Tl	0	0	0	NA	0	0	0	NA	0	0	0	0	0	0	0	0	0	NA	0	NA
U	2	6	1	0	5	34	0	1	1	8	1	2	1	4	0	8	0	NA	0	0
V	15	22	8	8	11	16	7	6	16	44	10	33	17	12	7	24	3	2	7	7
W	NA	NA	NA	NA	NA	1	NA	NA	1	1	NA	1	NA	NA	NA	1	NA	NA	NA	NA
Y	13	36	3	3	15	65	2	3	4	29	6	9	7	24	3	44	1	0	1	3
Zn	220	20	55	10	54	17	37	6	129	27	39	38	110	20	48	14	33	18	43	9
Zr	15	30	7	7	11	18	6	5	11	39	17	138	16	20	6	39	3	2	9	5

SI Table 4 Extractable element means by site and depth n=3 for most samples. Elements below detection limits (mg/kg) include Ag 1, Be 0.05, Bi 0.3, Hg 2, In 0.05, Mo 3, Sn 0.7, Th 0.7, Tl 0.06, W 0.8, Zr 0.5. Distances (km) for sites are as follows: DL (6.8), LU (8.2), BL (13.6), CW (20.2), LL (20.5), BR (24.0), AS (26.5), MG (31.0), CL (50.1), CF(50.2).

Site	DL		LU		BL		CW		LL		RC		AS		MG		CL		CF	
	Middle	Deep	Middle	Deep	Middle	Deep	Middle	Deep	Middle	Deep	Middle	Deep	Middle	Deep	Middle	Deep	Middle	Deep	Middle	Deep
Al.Li	19	34	7	6	33	8	11	10	14	21	40	17	9	NA	42	17	4	7	19	3
As.Li	2	NA	2	0	1	0	4	0	1	0	0	0	1	0	1	0	0	NA	0	NA
B.Li	3	3	2	3	3	3	2	3	3	3	3	1	3	2	5	2	6	5	4	2
Ba.Li	4	3	6	6	8	4	4	5	6	3	11	3	8	5	10	4	11	15	10	6
Ca.Li	848	511	550	812	366	629	445	329	552	582	238	96	494	455	466	252	246	337	348	210
Cd.Li	0	NA	NA	NA	0	NA	0	NA	0	NA	0	NA	0	NA	0	NA	NA	NA	0	NA
Co.Li	2	0	1	0	1	0	0	0	1	0	1	0	1	0	1	0	0	0	0	0
Cr.Li	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0	1	NA
Cu.Li	1	0	0	NA	1	NA	2	NA	2	0	0	0	0	NA	1	0	NA	NA	NA	NA
Fe.Li	135	89	70	103	41	75	55	35	76	88	53	25	56	53	99	50	9	23	40	16
K.Li	24	32	44	38	79	22	88	82	114	32	93	17	152	20	256	22	306	74	277	34
Mg.Li	290	202	99	182	107	215	99	142	171	213	92	30	163	159	177	96	155	167	209	101
Mn.Li	9	0	4	1	11	3	3	2	8	2	10	2	16	2	9	1	1	0	4	1
Na.Li	171	145	153	217	183	173	242	276	196	148	163	95	187	117	299	145	440	359	287	182
Ni.Li	86	0	12	0	33	0	10	0	13	0	8	0	5	0	7	0	1	0	1	NA
P.Li	NA	NA	NA	NA	NA	NA	15	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Pb.Li	0	NA	NA	NA	NA	NA	0	NA	0	NA	0	NA	0	NA	0	NA	NA	NA	0	NA
Rb.Li	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	1	0
Sb.Li	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sc.Li	NA	0	0	0	0	0	NA	0	0	0	0	0	NA	0	0	0	0	NA	0	0
Se.Li	NA	NA	NA	NA	NA	NA	NA	0	0	NA	NA	NA	NA	NA	NA	NA	NA	1	NA	NA
Sr.Li	4	2	2	2	2	3	2	1	3	3	2	1	2	2	3	1	2	3	2	1
Ti.Li	0	1	0	1	0	0	0	0	1	1	NA	NA	1	0	NA	NA	1	1	1	0
U.Li	NA	NA	NA	NA	NA	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
V.Li	NA	0	NA	0	NA	0	0	NA	0	1	NA	0	NA	0	NA	0	NA	NA	0	NA
Y.Li	NA	0	NA	NA	0	0	NA	0	0	0	NA	0	NA	NA	NA	0	NA	NA	NA	NA
Zn.Li	14	3	7	5	10	4	7	6	10	3	9	4	9	4	12	5	14	12	11	6

SI Table 5 Enrichment and media preparations. Peat media as well as amendment and growth substrate concentrations are presented here. Components indicate individual chemicals in a solution and preparation briefly describes the process for making each amendment of substrate.

Peat Media	1000x Stock Concentrations (mg/L)	Final Media Concentrations (mg/L)
KCl	1500	1.5
KH_2PO_4	13600	13.6
NH_4Cl	26800	26.8
$CoCl_2*6H_2O$	24	0.024
$ZnCl_2$	75	0.075
H_3BO_3	19	0.019
$NiCl_2*6H_2O$	24	0.024
$Na_2Mo_4*2H_2O$	24	0.024
$FeCl_2*4H_2O$	1344	1.344
$MnSO_4*4H_2O$	26	0.026
$MgSO_4*7H_2O$	1556	1.556
$CaCl_2*2H_2O$	2336	2.336
$CuSO_4*5H_2O$	9	0.009
$AlK(SO_4)_2*12H_2O$	3446	3.446

Amendments	Stock Concentration	Final Concentrations	Components	Preparation
TiNta	83 mM	0.83 mM	7.2 mL 1 M Tris pH 8, 4.8 mL NaNta, 0.55 mL $TiCl_3$	Bubble solutions, mix and filter sterilize in glovebox
CoM	0.2 M	2 mM	2-Mercapthoethanesufonic acid	Bubble, autoclave
Yeast Extract	0.40%	0.4 mg/L	Yeast Extract	Mix and bubble, filter sterilize in glove box
MES	6 and 6.75 pH	5 mM	2-(*N*-morpholino)ethanesulfonic acid	Mix and bubble, filter sterilize in glove box
Vitamins	1x	0.2, 1.0, 0.5, or 0.01 mg/L	100x stock (mg/mL): 20 biotin, 20 folic acid, 100 pyridoxine hydrochloride, 50 riboflavin, 50 thiamine hydrochloride, 50 nicotinic acid, 50 DL-calcium pantothenate, 1 vitamin B_{12}, 50 *p*-aminobenzoic acid, 50 lipoic acid	Mix and bubble, filter sterilize in glove box
Homopipes	5.4 pH	5 mM	Homopiperazine-1,4-bis(2-ethanesulfonic acid)	Mix and bubble, filter sterilize in glove box
Sulfide	0.4, 0.04 mM	4, .4 µM	Na_2S	Mix and bubble, filter sterilize in glove box
Rifampicin	1 mg/mL	0.01 mg/mL	Rifampicin	Mix and bubble, filter sterilize in glove box
Ampicillin	10 mg/mL	0.1 mg/mL	Ampicillin	Mix and bubble, filter sterilize in glove box
Growth Substrates				
Methanol	0.25 M	2.5 mM	Methanol	Bubble then add substrate, filter sterilize in glove box
Acetate	0.05 M	0.5 mM	Acetate	Bubble then add substrate, autoclave
Ethanol	0.25 M	2.5 mM	Ethanol	Bubble then add substrate, filter sterilize in glove box
Butanoate	1 M	10 mM	Boric Acid	Bubble then add substrate, filter sterilize in glove box
Propionate	1 M	10 mM	Propionic Acid	Bubble then add substrate, filter sterilize in glove box
Glucose	1 M	10 mM	Glucose	Bubble then add substrate, filter sterilize in glove box
TMA	0.25 M	2.5 mM	Trimethylamine	Bubble then add substrate, filter sterilize in glove box